The Role of MicroRNAs in the Pathophysiology and Diagnosis of Non-alcoholic Fatty Liver Disease

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ABBREVIATIONS

3'UTR	3' untranslated region
AASLD	American Association for the Study of Liver Diseases
ABCA1/ ABCG1	adenosine triphosphate binding cassette A1/G1
Ago2	argonaute2
ALD	alcoholic fatty liver disease
ALT	alanine aminotransferase
AMP	adenosine mono-phoshate
AMPKα	AMP kinase subunit-α
ANP32A	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
APOA1	apolipoprotein A1
ASH	alcoholic steatohepatitis
AST	aspartate aminotransferase
AUROC	Area under ROC
BMI	body mass index
C/EBPα/β	CCAAT/enhancer-binding protein alpha/ beta
CK-18	cytokeratin-18
CoA	coenzyme A
CPT1a	carnitine palmitoyltransferase 1A
CROT	carnitine O-octaniltransferase
CVD	cardiovascular disease

DGCR8	DiGeorge syndrome chromosomal [or critical] region 8
Dhcr7	7-dehydrocholesterolreductase
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Exp5	exportin 5
FABP7	fatty acid-binding protein 7
FAS	fatty acid synthase
FASN	gene of fatty acid synthase
FFA	free fatty acid
FLD	fatty liver disease
FXR	farnesoid X receptor
HADHB	hydroxyacyl-CoA-dehydrogenase
HBV/ HCV	hepatitis B/C virus
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HFD	hypercaloric diet enriched in fat
HFD-HFr	hypercaloric diet enriched in fat and fructose
HIV	human immunodeficiency virus
Hmgcr	3-hydroxy-3-methylglutaryl-Coa reductase
Hmgcs1	3-hydroxy-3-methylglutaryl-Coa synthase 1
IHH	immortalized human hepatocytes
IL-1RA	interleukin 1 receptor antagonist
IRS2	insulin receptor substrate 2
JNK	JUN N-terminal kinase

JUN	gene of C-JUN
LSDP5	lipid storage droplet protein5
LXR	liver X receptor
MetS	metabolic syndrome
miRNA/ miR	microRNA
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NAFL	non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa beta
NPC1	endolysosomal transport protein Niemann-Pick C1
OA	oleic acid
PAMP	pathogen-associated molecular patterns
PLIN5	perilipin5
PPAR	peroxisome proliferator-activated receptors
PTEN	phosphatase and tensin homolog
PXR	pregnane X receptor
RASSF1A	Ras association domain family 1 isoform A
RISC	RNA-induced silencing complex
RNA	ribonucleic acids
RNAi	RNA interference
RNase	ribonuclease

RNP	ribonucleoprotein
ROC	receiver operating characteristics
siRNA	small interfering RNA
SIRT1/6	sirtuin 1/6
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, and member 4
snRNP	small nuclear RNP
SR-BI	hepatic scavenger receptor class B type I
SREBP-1c	sterol regulatory element binding protein 1-c
T2DM	type 2 diabetes mellitus
TGFBR2	TGFβ-receptor 2
TGFβ	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
VLDL	very low density lipoprotein
WAT	white adipose tissue

SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is a growing health problem in Western countries. Clinically bland steatosis (NAFL) and the more severe form of non-alcoholic steatohepatitis (NASH) have to be discriminated. The pathophysiological mechanisms responsible for the development and progression of NAFLD remain widely unclear. In clinical practice, the development of non-invasive diagnostic tools to differentiate between NAFL and NASH would be desirable for optimal follow-up and treatment of NAFLD patients.

As microRNAs (miRNAs) are already used in different clinical settings for early disease detection and monitoring of disease progression, their potential to function as diagnostic tool for NAFLD was evaluated in human serum samples. In two independent human patient cohorts, the levels of four miRNAs were evaluated to find characteristic patterns. MiRNA 21 was found to be significantly different regulated in NASH patients only. As two other miRNAs with a known prognostic potential could be validated in our study, the diagnostic ability was evaluated in a combined approach. The combination of miRNA expression with apoptosis marker CK-18 fragment level in a simplified scoring system showed excellence in comparison to other prognostic tools such as the level of transaminases.

In the second part of this study, the aim was to define miRNAs involved in the pathophysiological mechanisms of NAFLD. MiRNAs with characteristic expression patterns as well as potential target genes were investigated in a human biopsy cohort. Also, an in vitro model of human hepatocytes simulating the conditions of hepatic lipid accumulation and lipotoxicity was investigated to detect parallels in miRNA and target

gene expression patterns compared to patient samples. Expression of miR 223 and miR 638 as well as of target genes JUN, FASN and PLIN5 in cultured cells showed similarities to human data.

The significant difference in regulation of miRNA 122 between NAFL and NASH patient group indicates a potential regulative function of the miRNA in disease pathophysiology. A putative pathogenetic factor in disease development could be also the newly identified association between NAFLD and miRNA 638, which has a predicted key-role in the pathway of lipid accumulation. The inverse correlation of expression of the miRNA and its potential target gene Perilipin 5 suggests a regulatory interaction and is an important finding in the context of defining relevant mediators in the pathophysiology of NAFLD.

ZUSAMMENFASSUNG

Die nicht-alkoholische Fettlebererkrankung (NAFLD) stellt eine zunehmende Gesundheitsgefährdung dar. Allgemein muss zwischen einfacher Steatose (NAFL) und der schwereren Form der Steatohepatitis (NASH) unterschieden werden. Die pathophysiologischen Mechanismen bei der Entstehung und Progression von NAFLD sind noch weitgehend ungeklärt. In der klinischen Praxis wird die Entwicklung von nicht-invasiven diagnostischen Hilfsmitteln zur Unterscheidung zwischen NAFL und NASH angestrebt, um eine optimale Behandlung und Betreuung von NAFLD Patienten zu gewährleisten.

Da MikroRNAs (miRNAs) bereits im Bereich der diagnostischen Erkennung als auch in der Überwachung von Krankheitsverläufen zur Anwendung kommen, wurde ihr Potential als diagnostisches Hilfsmittel im Serum von NAFLD Patienten untersucht. In zwei unabhängigen Patientengruppen wurde das Vorkommen von vier MikroRNAs auf mögliche charakteristische Muster hin untersucht. Im Serum der NASH Patienten konnte so eine signifikant höhere Konzentration von miR 21 nachgewiesen werden. Da zwei weitere miRNAs mit einem bereits bekannten prognostischen Potential durch die vorliegende Studie validiert werden konnten, wurde die Verwendung dieser drei miRNAs als diagnostischer Marker in einem kombinierten Ansatz überprüft: Die Verbindung der miRNA-Level mit dem Apoptosemarker CK-18 in einem vereinfachten Bewertungssystem stellte im Vergleich zu bekannten prognostischen Hilfsmitteln wie Serumtransaminasen eine Verbesserung dar.

Ein weiteres Ziel dieser Studie war es, Assoziationen von miRNAs mit den pathophysiologischen Mechanismen von NAFLD zu definieren. Sowohl miRNAs mit

charakteristischen Expressionsmustern als auch deren potentielle Zielgene wurden in einer Kohorte aus humanen Leberbiopsien untersucht. In einem in vitro Model wurde versucht Parallelen von miRNA- und Zielgenexpression zu den Biopsieproben ausfindig zu machen, welche im Fall der miRNAs 223 und 638 sowie der Gene JUN, FASN und PLIN5 erfolgreich nachgewiesen werden konnten.

Durch den neubeobachteten Unterschied in der Expression von miR 122 zwischen NASH und NAFL Patienten kann eine aktive regulatorische Rolle der miRNA in der Pathophysiologie von NAFLD angenommen werden. Ein potentieller Zusammenhang besteht auch zwischen der Manifestierung der Krankheit und der in dieser Studie neu mit ihr assoziierten miR 638, der eine Schlüsselrolle im Signalweg der Lipidakkumulation eingeräumt wird. Die inverse Korrelation der Expression der miRNA und ihrem potentiellen Zielgen PLIN5 geben Hinweis auf eine mögliche regulatorische Interaktion und sind eine wichtige Erkenntnis vor dem angestrebten Ziel, relevante Mediatoren in der Pathophysiologie von NAFLD zu definieren.

1 INTRODUCTION

1.1 The Metabolic Syndrome

The metabolic syndrome (MetS) is defined as an accumulation of health risk factors for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). It is an increasing health problem worldwide[1]. The manifold factors include central obesity, elevated blood pressure, impaired glucose tolerance or dysglycemia, increased triglyceride levels and decreased high density lipoprotein (HDL) cholesterol levels. But also, the syndrome is associated with other clinical conditions besides from T2DM and CVD. Those include chronic low-grade inflammation, oxidative stress, hyperuricemia, hypertension, dyslipidemia, hyperandrogenism, hypogonadism and polycystic ovary syndrome, obstructive sleep apnea, vascular dementia and Alzheimer's disease, certain forms of cancer, hepatic steatosis and non-alcoholic fatty liver disease (NAFLD)[2-4]. The latter will be in the main focus of the following thesis. When Reaven first described the current paradigm of the MetS in 1988, he used the term *Syndrome X*, describing the interaction and relation between insulin resistance, hypertension, T2DM and CVD[2]. Up from then, many different terms found their way into medical literature, including the *deadly quartet*, the *cardiometabolic syndrome*, and the *insulin resistance syndrome*[4]. As MetS is a multifactorial disease summarizing always more than one component, there are many existing cutoffs for the thresholds in diagnostic parameters, which lead to the

condition that different medical boards have set up and established different criteria for MetS diagnosis during the last decades. Unaffected by these fine differences in the definition of diagnosis, it is a fact that a clustering of health factors for CVD, including obesity, has been observed and described already in the beginning of the 20th century[5].

An epidemiological study recently published confirmed one third of the adult population of the USA is affected by MetS[1]. The syndrome is responsible for the majority of health care expenditures in the United States, which had the consequence to classify MetS to be more threatening to world health than acute infectious diseases as e. g. the human immunodeficiency virus (HIV) for developed countries[6, 7]. However, MetS is not just a problem of the obese population. For instance, 20 % of people with morbid obese (body mass index (BMI) > 40 kg/m^2; > 35 kg/m^2 with comorbidities) health status have normal life spans and are, by definition, metabolically healthy[8, 9]. On the contrary, 40% of the population with normal BMI suffers from pathologies as hypertension, dyslipidemia, CVD and also NAFLD[10, 11]. These numbers show that MetS is not only caused by obesity, but still it remains *the* major health risk factor.

1.2 Non-alcoholic fatty liver disease

Fatty liver disease (FLD) is describing a broad spectrum of hepatic, metabolic as well as histological disorders and is characterized with a chronic and aberrant accumulation of triglyceride droplets in >5 percent of liver cells. Major causes of this

disorder are abusive consumption of alcohol (alcoholic fatty liver disease (ALD))[12], an infection by genotype 3 hepatitis C virus or metabolic disorders which are associated with obesity. In particular, this metabolic manifestation is defined as non-alcoholic fatty liver disease[13]. There is only little known about the pathophysiological background and the progression of NAFLD. Also, no reliable non-invasive diagnostic marker for the diagnosis of the different states of NAFLD has been defined. One key aspect of the work in hand is to investigate molecular factors triggering the manifestation and progression of NAFLD. The other is to evaluate potential biomarkers for non-alcoholic fatty liver disease in the context of the metabolic syndrome. The main focus for the two aims is on small non-coding ribonucleic acids (RNAs) – so-called microRNAs.

1.2.1 Definition, epidemiology and natural course of NAFLD

The term “NAFLD” is often used as a summary term in the context of other clinical terms and conditions: bland/simple steatosis or non-alcoholic fatty liver (NAFL), non-alcoholic liver inflammation or non-alcoholic steatohepatitis (NASH) – many different names which may cause confusion in clinical practice as well as for patients. To avoid those and to set up diagnostic thresholds, the American Association for the Study of Liver Diseases (AASLD) established a diagnostic guideline. According to this, NAFLD diagnosis requires a primary accumulation of lipids with an exclusion of secondary causes for hepatic fat accumulation (e.g. extensive alcohol abuse,

steatogenic medication, viral infections and hereditary disorders)[14]. This lipid accumulation is also called hepatic steatosis and is defined as cytoplasmatic macro-, micro- or mixed vesicular incorporation of lipids within the hepatocytes[15]. In this condition medical literature coined the terms bland or simple steatosis or NAFL. If NAFL is progressing into inflammation and hepatocellular injury, it has to be separated as the more severe form of the disease, non-alcoholic steatohepatitis (NASH). By definition, NASH is a combination of primary steatosis and inflammation with hepatic injury; such is ballooning and necroapoptosis of hepatocytes.

So summarized, the whole condition of NAFLD is separated into NAFL and NASH (see also Figure 1-1).

The factor making NAFLD to a dangerous health condition is that NAFL patients might develop NASH, which leads to an increased risk to develop into fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[18, 19]. These factors are causing a significant decrease in over-all survival for NASH patients (survival 70% versus 80%, mean observational interval 13.7 years) in comparison to a control population (risk factor adjusted) and to NAFL patients[20, 21].

NAFLD is an increasing epidemic health disorder in western countries. In Europe, it is the most common source of chronic liver disease[22, 23]. The prevalence is currently 20% to 35% of adult population in developed countries, summiting in up to 50% of patients in the USA[24, 25]. At the same time NAFLD is gaining a health problem for children with an increasing prevalence ranging between 13% and 80%, which is strongly correlated to the manifestation of obesity[26].

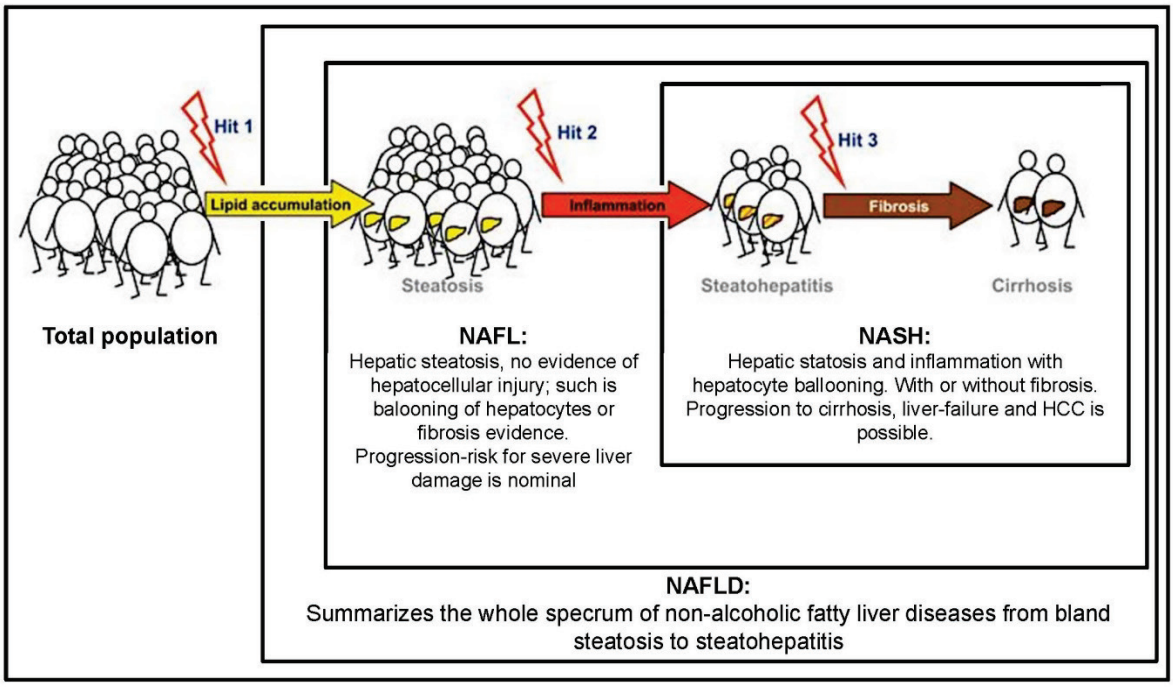

Figure 1-1: Natural course and spectrum of NAFLD in total population (reproduced from[14, 16, 17])

It is well known that NAFLD is strongly associated with the risk factors of the metabolic syndrome which are addressed in chapter 1.2 of this work. The prevalence of obesity in NAFLD patients is between 30% and 100%, patients suffering from an additional T2DM are between 10% to 75%, still[27]. Assy et al. addressed in a study, that 50% of hyperlipidemia patients could be proven with hepatic fat infiltration by ultrasonography[28]. In another study, insulin resistance could be shown as a predictor for NASH (prevalence of 60%), evaluated in patients undergoing gastric bypass surgery[29]. As obesity is a well-established risk factor for NAFLD, characteristics of obese patients have been evaluated into depth. It has been shown that morbid obesity patients who are also undergoing bariatric surgery

are exceeding the level of 90% of NAFLD[14]; 12% of patients in this group were even suffering from advanced fibrosis in the liver[30, 31].

In the progression of NAFLD, 5% to 20% develop NASH; where-in 5% to 20% proceed into the development of fibrosis of higher grade. A small percentage (<5%) of those develop the clinical condition of a liver cirrhosis[32]. Estimations about the progression rates of NAFL patients into cirrhosis have also been performed; the calculated prevalence of NAFLD-cirrhosis in total population is between 0.05% and 0.3%[33]. These numbers show that patients suffering from NAFLD carry an increased risk to develop severe liver disease. Remarkably, Ertle et al. found cases of HCC even in pre-cirrhotic livers, nevertheless highly correlated with the presence of advanced fibrosis and cirrhosis[34]. Following international estimations about the rapidly increasing numbers of NAFLD patients worldwide, it is already assumed, that NAFLD related HCC rate will also increase and will be doubled within the next decade[35].

Simplified, one can say that NASH patients have an elevated mortality rate; but NAFL patients do not[14].

Liver related mortality is on the third most common cause of death worldwide (13%) only exceeded by malignancies and CVD[36]. For obvious reasons the pathophysiological background as also the diagnostic methods deserve further investigation, as it is addressed in the context of this work.

1.2.2 Pathophysiology of NAFL and NASH

The liver holds a central position in the context of glucose- and lipid-metabolism. The excess deposition and accumulation of lipid droplets in liver cells can cause adverse effects in the liver as well as in the whole organism. The physiological manifestation of NAFLD can be basically explained as an elevation of free fatty acid (FFA) inflow within the hepatocytes.

The source of these FFA is an increase of hepatic de novo lipogenesis, an impaired lipid export out of the hepatocytes, an alteration of hepatic processing of dietary lipids delivered by lipoproteins or they are derived from insulin resistant adipose tissue[37]. As addressed under 1.2.1 in this work, a fraction of patients with bland steatosis proceed into the development of hepatocellular damage and inflammation defined as NASH.

The most common paradigm describing the progression of NAFLD from simple steatosis to NASH is the ‘two-hit-hypothesis’ by Day et al. The ‘first hit’ described an imbalance of cellular lipid uptake or combustion, leading to hepatocellular lipid accumulation (NAFL). The imbalance between pro- and anti-inflammatory factors represents the ‘second hit’ which is defined by additional inflammation and leads to the development of NASH[17]. In Figure 1-1 those hits are also addressed. Bosserhoff and Hellerbrand extended Days’ paradigm by a ‘third hit’, which is defined by the imbalance of pro- and anti-fibrotic factors and leads into progressive hepatic fibrosis, ultimate cirrhosis and HCC[16].

In the following, selected pathways and receptors involved in the pathophysiology of NAFLD will be introduced. A detailed discussion for selected genes of capital importance will be addressed later in this thesis in chapter 1.4.

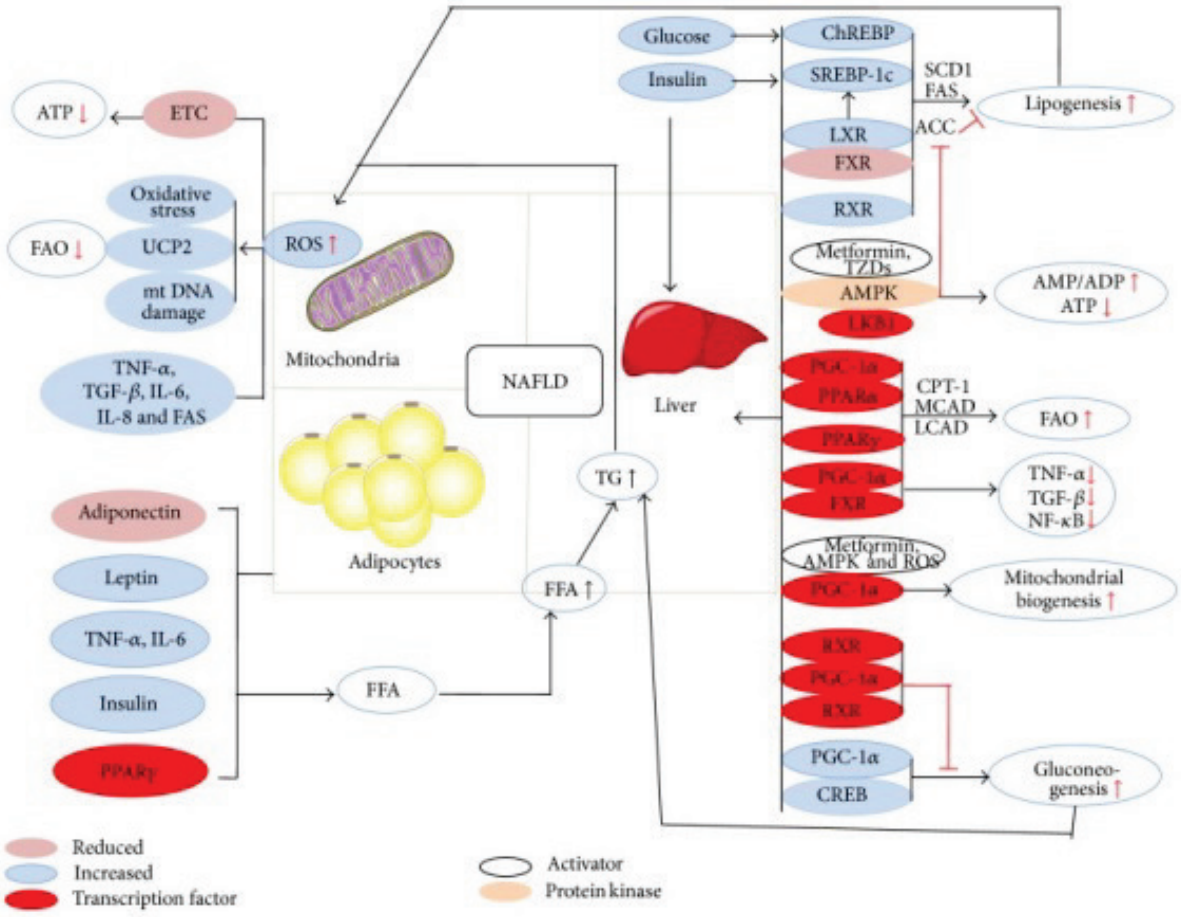

Figure 1-2: Relevant pathways in the development of NAFLD (reproduced from [38])

The first interaction to mention in the context of MetS and NAFLD is the clear association with insulin resistance. In the majority of NAFLD patients an impaired insulin signaling is occurring. Both are described in the context of pathophysiology for each other. It remains unclear whether insulin resistance is causing NAFLD or it

is one of its consequences[38]. Insulin is physiologically involved in lipogenesis and glucose uptake. When it comes to insulin resistance, the glucose uptake in the liver triggered by insulin is decreasing. Glucokinase activation is the major mechanism of insulin in glycolysis stimulation[39]. Insulin is also described as an activator for sterol regulatory element binding protein 1-c (SREBP-1c). This transcription factor is responsible for the stimulation of fatty acid synthase, acetyl-coenzyme A (CoA) carboxylase, liver pyruvate kinase and stearoyl-CoA desaturase; all known as key mediators in fatty acid synthesis[40]. The ability of insulin to increase lipogenesis might lead to the assumption that a resistance might cause an impaired fatty acid synthesis. However, it has been shown that in a state of high insulin resistance, insulins' ability to activate SREBP-1c and promote lipogenesis is still active[41], even when insulin is no longer able to decrease glucose production[42]. Insulin resistance in dysfunctional adipocytes is leading to an elevated level of released FFAs and taken up in distant tissues, such is the liver. An accumulation of triglyceride-derived toxic lipid metabolites within the hepatocytes and Kupffer cells is the consequence, which on their side again may cause an activation of inflammatory pathways[43].

The next important compartments to mention in this context are Peroxisome Proliferator-Activated Receptors (PPARs). PPARα, one of three known isoforms, is described as an important mediator in hepatic lipid balance. They can cause an increase of beta oxidation and are activated by fatty acid binding[44]. A correlation between the decrease of PPARα and an increase of NAFL has been published by Yeon et al[45]. The described activation of fatty acid beta oxidation can cause a

decrease in glucose uptake. This was shown in a study with diabetic patients and rodents, where PPARα overexpression caused an elevated glucose level[46]. The ability of PPARα to decrease plasma triglyceride levels largely by increasing triglyceride clearance, its role in regulating the availability of triglycerides for Very Low Density Lipoprotein (VLDL) secretion as well as the contribution in lowering tumor necrosis factor alpha (TNF-α) levels are all effects showing the contribution of PPARα to the pathophysiology of NAFLD[47, 48].

In the context of lipid absorption, it is important to mention the role of bile acids. These steroid acids synthesized by the liver are involved also in signaling and the uptake of fat soluble vitamins[49]. A critical role of bile acids is their function as a ligand for the Farnesoid X Receptor (FXR) which is involved in various inflammatory pathways, glucose and lipid homeostasis[50, 51]. It has been shown that FXR is similar to PPARα involved in activation of fatty acid beta oxidation. SREBP-1c, the transcription factor activated by insulin[40], is inhibited by FXR - which may cause a downregulation of lipogenesis rates[52]. A study showed decreased expression of FXR in NAFLD patients, correlated with an increase of SREBP-1c and the Liver X receptor (LXR)[53]. In the context of NAFLD it is also important to clarify that FXR is playing a crucial role in antagonism of the nuclear factor kappa beta (NF-κB) signaling pathway, as this has been described to reduce hepatic inflammation[54]. These examples are showing the important role of FXR in the context of NAFLD, and might explain the beneficial effect of bile acids on NAFLD patients by FXR activation.

Liver X receptors are transcription factors involved in the control of lipid homeostasis by binding to specified LXR-response elements[55]. As cholesterol sensors, they are involved in the metabolism and absorption of sterols. A study described their promoting role in the development of NASH by an indirect activation of SREBP-1c[56]. Joseph et al. showed a direct involvement in regulation of lipid synthesis, by interacting with genes such as for instance fatty acid synthase (FAS)[57].The direct connection of LXR to NAFLD has been already addressed above; a study has shown an increase of LXR correlated with an increase in SREBP-1c expression in the liver[53]. Former investigations could also demonstrate a connection between the redox state of the cell and LXR, specifically as a reaction partner in the oxysterol synthesis, which have an elevated level in NAFLD patients and act as important LXR activators[58-60]. First approaches about a reduction of *reactive oxygen species* production - another precursor unit of oxysterol synthesis - to reduce hepatic steatosis by decreasing the LXR level are currently under investigation. Only these few facts can already clarify the capital role LXR is playing in the pathophysiology of NAFLD.

Another well-known receptor group in the context of NAFLD is the Pregnane X Receptor (PXR) family. Those classical hormone and xenobiotic receptors have been described in the context of metabolism and detoxification, specifically as stimulators of lipogenesis. Their high expression in the liver as well as the ability of decreasing fatty acid oxidation and gluconeogenesis is showing the important role in metabolism[38, 61]: The knockout of PXR in a diabetic mouse model causes an

inhibition of lipogenesis, an elevation in mitochondrial fatty acid beta-oxidation, an enhancement in insulin signaling and lower levels of inflammatory markers[62]. PXR represents another therapeutic target in the context of NAFLD; hence, developing and investigating potential agonists has already been started.

As it has been addressed to be connected to numerous of the shown pathways and receptors, the role of SREBP-1c will be discussed in more detail in the following. Multiple interactions in the context of metabolism and NAFLD[40, 41, 52, 53] are distinguishing its importance. Activated by insulin, SREBP-1c itself has an activating role at several points of the lipogenesis pathway. In several studies concerning SREBP-1c contribution in NAFLD, increased expression levels could be detected. An indirect cause of this is the upregulation of fatty acid oxidation by relieving the inhibition of one of its enhancing proteins, Forkhead box protein A2[63]. An elevated rate of fatty acid beta-oxidation, an increase in lipogenesis as also the increase in mitochondrial *reactive oxygen species* production fit well the hypothesis that SREBP-1c is playing a key-role as activator and mediator in NAFLD which could also be shown in in vitro studies[64, 65].

These interactions are also promising factors to serve as targets in therapeutic approaches of SREBP-1c inhibition, leading to a potential prevention from liver steatosis and a decreased level of insulin resistance in a high-fat diet rodent model[66]. Pharmacologic modulation of SREBP-1c by the Japanese herbal ingredient Bofutsushosan showed a prominent impact on several metabolic pathways, as the

decrease of the protein level also attenuated the progression from bland steatosis to NASH in mice[67].

To describe the complex network of involved pathways and receptors involved in the pathophysiology of NAFLD might go beyond the scope of this work. The selected samples give a glance about the close interaction between the main factors mediating the development of hepatic steatosis and steatohepatitis.

1.2.3 NAFLD – consequences and prognosis

To see the epidemiology and pathophysiology of NAFLD in the context of the entire organism, the following part will discuss the long-term outcomes as well as the intra- and extra-hepatic consequences of the disease.

It has already been addressed earlier, that NAFLD patients have an elevated mortality rate. This can be specified by the fact, that NASH patients mortality is increased in comparison to NAFL patients, as a significantly higher rate of cardiovascular and hepatic complications was detected[20, 21]. The progression into NASH is always implicating the presence of inflammation and fibrosis, which is often leading into the development of cirrhosis and cancer, and therefore is correlating with an elevated mortality[21]. An insight into the systemic consequences of hepatic lipid accumulation could be gained in context of the affected pathways and receptors (1.2.2) with the major outcome of an impaired insulin sensitivity causing metabolic

complications and increasing the risk for cardiovascular and hepatic events. It has been shown that the liver is not only affected by MetS passively. In fact, the onset of steatohepatitis and associated insulin resistance are causing a menacing cycle. Even if the total number of patients developing an end-stage liver disease is proportionally low, extra-hepatic pathologies of the MetS will be enhanced anyway[68]. A close correlation between NAFLD and the inflammatory cascade of atherosclerosis has been investigated, as also a correlation to the onset of kidney and cardiovascular disease in the context of NAFLD was in the focus of numerous studies[69]. The issue whether insulin resistance is a consequence or a cause of NAFLD has already been addressed. The fact that the exceeded lipid content of liver cells is responsible for a reduction in insulin sensitivity in hyperinsulinemia is supporting the causal role of NAFLD, as this is described as a known symptom of pre-diabetes[70]. The outcome of several studies showing an earlier onset of steatosis and insulin resistance in liver tissue compared to peripheral insulin resistance also points towards a primary causal effect of NAFLD in the development of T2DM and the MetS[71, 72].

1.2.4 Diagnosis of NASH versus NAFL

The differential diagnosis of NAFLD is a critical challenge. The most important step within the diagnostic pathway is the assessment of patients' alcohol consumption. It is crucial to detect prior or active consumption of relevant amounts of alcohol intake. As patients often conceal or underestimate their consumer habits, false diagnosis of

alcoholic fatty liver disease and alcoholic steatohepatitis (ASH) is always a risk. There are different thresholds for the amount of alcohol intake defined as hepatotoxic. The recommendations of the AASLD define >210 grams in average per week in men and >140 grams in average in women as the threshold for significant alcohol intake in clinical practice[14], whereas these values should be seen in a two-year timeframe before consultation and diagnosis.

Overall, one can say that all individuals suffering from obesity or any other manifestations of the Mets should be screened for NAFLD. But NAFLD is not restricted to these patients. Also individuals showing no symptoms in these pathologies have been detected with severe hepatic lipid accumulations.

Most NAFLD patients are asymptotic; however, sometimes symptoms as pressure in the epigastric area or elevated lassitude appear. As shown in Figure 1-3, in the most cases, the disease is detected by routine blood tests. A persistent elevation of liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is usually the first sign for a NAFLD manifestation and can be detected in 80% of NASH patients and in 50% of NAFL patients[73, 74]. It is also possible to detect liver steatosis by ultrasound in a clinical routine examination by an enhanced echogenicity. As it has been addressed earlier in concern of the alcohol consumption, an in-depth anamnesis is crucial. First of all, all other causes of liver diseases, causing elevated liver enzymes or hepatic steatosis should be evaluated and excluded. Examples could be medications or toxins, inflammatory or

autoimmune diseases, lipodystrophy and malnutrition, but also viral hepatitis should be taken into consideration. A next step would be the consideration of risk factors (as shown in Figure 1-3) making NAFLD more likely (e.g. T2DM and impaired glucose tolerance, obesity, hyperuricemia, arterial hypertension, hypertriglyceridemia and hypercholesterolaemia)[37].

In routine laboratory investigation the focus is on transaminase detection first. It is important to mention that serum transaminase levels will not infrequently be within the normal range in NAFLD patients; a problem strongly associated with one of the basic questions of this project. It also has to be mentioned that other serum parameters, such as ɣ-glutamyltransferase (GGT), cholinesterase or ferritin might be elevated as well, and also normal readings in laboratory chemistry do not totally exclude liver disease or even cirrhosis, as also some parameters are 're-normalized' during the progression into NASH[75]. All this leads to the conclusion that the diagnosis of NAFLD by routine parameters is not reaching satisfying performance levels. This statement is based on the consideration of sensitivity, specificity and the discrimination from other liver diseases and follows the recommendations of the AASLD[14, 37].

For diagnosis of hepatic steatosis, without differentiating between NAFL, NASH and even early cirrhosis[76], ultrasonography is the current gold standard[77]. Also computed tomography's potential is in detection of steatosis, but is lacking the ability of differentiation. As a rather costly alternative with the additional patients risk due to the exposure to radiation, it is not a suitable tool for routine diagnostics[76]. The high

costal factor, the limited availability as well as the restricted ability of ultimate discrimination also holds true for magnetic resonance imaging[78].

FibroScan is also a method to access the status of liver fibrosis by detecting the hepatic stiffness level. It is also described as transient elastography and has been established in a wide spectrum of liver diseases. The principle is simple, measuring the degree of fibrosis by ultrasound. Recent studies have shown correlations between FibroScan results and histological results, so an exclusion or diagnosis of advanced severe fibrosis, detectable in the progression from NASH into cirrhosis is possible[79]. Recent developments enable the non-invasive quantification of liver fat by shear wave elastography (Fibroscan CAP system) in selected centers[80-83].

It has been stated that already the diagnosis of NAFLD is challenging. In clinical routine the more crucial diagnostic decision is the discrimination between bland steatosis and steatohepatitis. The definition of non-invasive serum markers is one of the major aims of the thesis in hand and will be discussed more detailed later in this chapter as well as under 3 First manuscript. In the following current approaches in NASH diagnosis will be addressed first.

The terms of NAFL and NASH are sometimes not exactly discriminated, which may cause confusion in clinical routine. The guidelines of the AASLD define that NAFLD is requiring the presence of primary hepatic steatosis[14]. This should be diagnosed via imaging or by histology. Also, reasons for secondary hepatic fat accumulation, such as steatogenic medication, virus infection or alcohol abuse should be excluded,

as it has been discussed in detail under 1.2.2. Primary hepatic steatosis is associated with cytoplasmic micro-, macro or mixed vesicular incorporation of lipids in the hepatocytes (NAFL)[15]. The progression of NAFL usually comes in form of bacterial translocation or elevated levels of pathogen-associated molecular patterns (PAMPs) in the portal vein and causes steatohepatitis[84].

The gold standard in NASH diagnosis in clinical practice to differentiate between NAFL and NASH is the histological examination of a piece of liver tissue (liver biopsy) as shown in Figure 1-3. Liver biopsy is usually performed through the skin under ultrasound guidance[85-87]. It involves taking a small liver section using a sharp hollow needle. This can be performed under local anesthesia[85-87]. Percutaneous biopsy bears an increased risk for patients health, as it can cause clinical significant bleeding (1.1-1.6%)[85, 86], which might be fatal[86]. The high prevalence of MetS in the population is making it unrealistic to apply the invasive procedure to all individuals at risk for a NAFLD manifestation. Even if liver biopsies are to date the only possibility to evaluate liver pathology, they are only reflecting the situation at a certain point of time. Furthermore, in-depth anamnesis of individuals remains crucial, to discriminate between NASH and ASH which is still not possible solely based on histology[88].

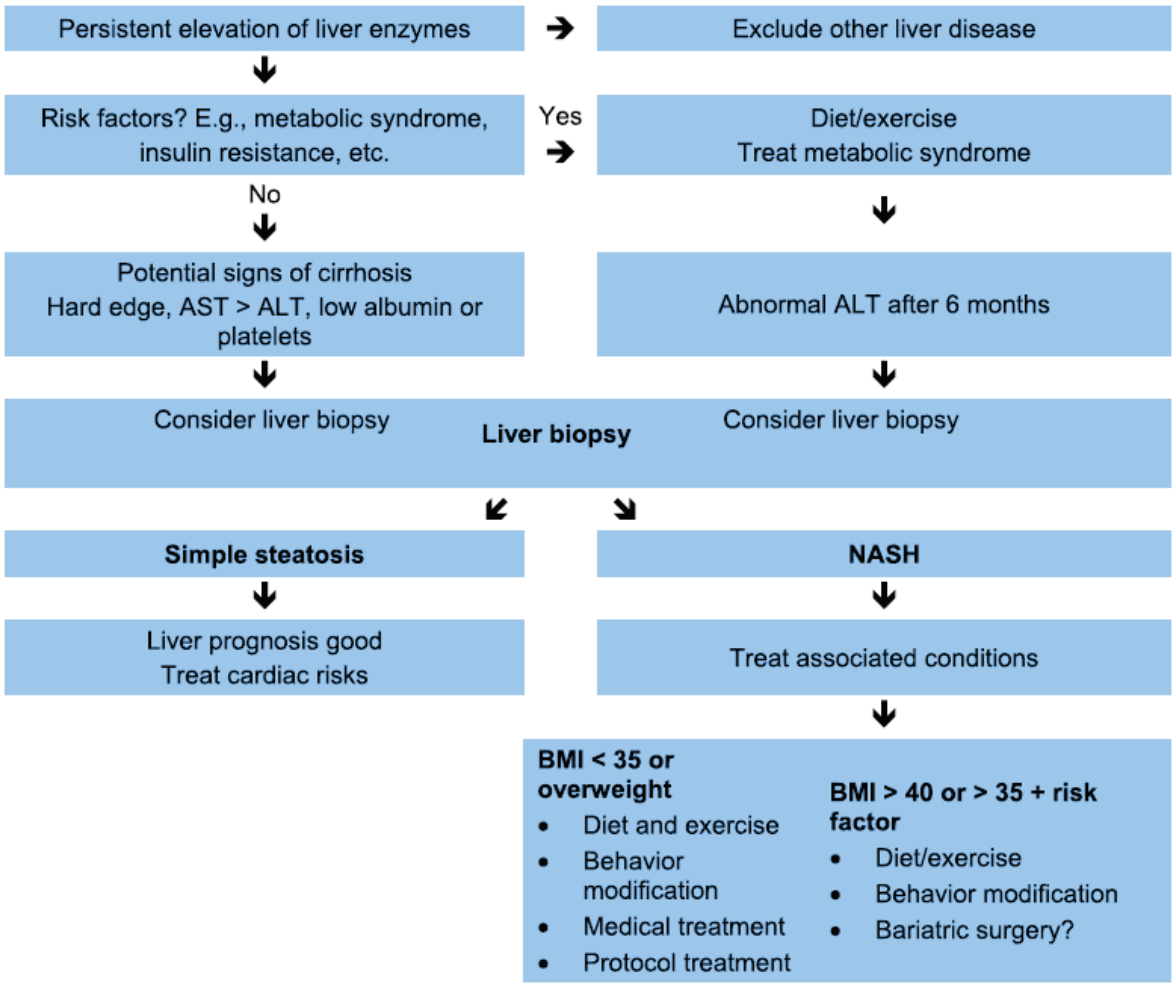

Figure 1-3. Management of fatty liver disease diagnosis (reproduced from [89, 90])

Taken together, by considering the risks and efforts linked to liver biopsies and the limited opportunities for the discrimination between 'simple' steatosis and steatohepatitis for the described imaging technologies - the development of non-invasive diagnostic tools to differentiate between NAFL and NASH remains crucial for optimal follow-up and treatment of NAFLD patients.

Alternative methods for NASH diagnosis have also been under investigation. A recently published breath test study investigated the compounds of volatile organic material in exhaled air to diagnose the presence of NASH. The procedure based on gas chromatography - mass spectrometry analysis, could show a significant number of additionally diagnosed NASH patients and could decrease the number of liver biopsies without necessity[91].

Nevertheless, the current focus in NASH diagnosis still remains on the establishment of non-invasive blood biomarkers. A main attribute of steatohepatitis is the increase of apoptosis of liver cells. During liver cell apoptosis, cytokeratin-18 (CK-18), an intermediary filament protein expressed by hepatocytes is which is proteolytically cleaved by caspases[92, 93]. The resultant CK-18 fragments are released in patients' blood by the hepatocytes and can be detected in serum by specific monoclonal CK-18-antibody M30 in an Enzyme-linked Immunosorbent assay (ELISA)[94]. CK-18 fragments are one of the most promising biomarkers for NASH diagnosis – one reason, why it has been in a special focus in the experimental part of the work in hand. Its appearance could also be correlated with histological progression of NAFLD[77]. However, the sensitivity and positive predictive value for NASH is low[95] and the AASLD practice guideline describes CK-18 fragments as a marker with a high potential, but not recommended as solid marker for differentiation between NAFL and NASH[14]. The search for reliable non-invasive biomarkers is still ongoing. Potential candidates, such as the interleukin 1 receptor antagonist (IL-1RA) have been investigated in the serum of NASH patients. Here, a

clear trend of upregulation during the progression of NAFLD could be shown, but still lacking on satisfying results in terms of NASH discrimination from NAFL[96]. Numerous other approaches have been investigated – fibroblast growth factor 21, TNF-α and other promising inflammatory markers – but the diagnostic performance and validation in independent cohorts have not lead to satisfying outcomes. One problem could be that other organs suffering from MetS are affected by inflammatory reactions also, which might be reflected by blood serum levels. In recently published studies the diagnostic potential of microRNAs, small RNAs of 19 to 23 nucleotides in total length regulating gene expression and usually acting at posttranscriptional level, have been investigated. These miRNAs, usually evaluated in cell free plasma or serum are also called circulating miRNAs. The range of miRNA applications is getting broader as they are used in different clinical settings for early disease detection and monitoring of disease progression[97]. The advantage of circulating miRNAs is obvious, as they give a less invasive opportunity to describe the physiological as well as the potential pathological state of their origin organ/ tissue. Very recently, Pirola et al. made an attractive approach by using miRNAs as potential diagnostic markers for the assessment of NAFLD[97]. The pooling of CK-18 and fibroblast growth factor 21 levels also increased specificity and sensitivity of NASH diagnosis[98, 99]. The combined knowledge of these approaches have put the basis for one main part on the experimental setting of this work, as the combination of miRNA expression level with CK-18 fragment level have been investigated in the first part of the project ‘Performance of Serum microRNAs -122, -192 and -21 as

Biomarkers in Patients with Non-Alcoholic Steatohepatitis' (see 3 First Manuscript). The diagnostic performance of acquired results had promising potential. However, to achieve a broad clinical application, the further investigation and improvement of NASH biomarkers remains crucial[98].

1.2.5 Treatment options

After a successful diagnosis of NAFLD, respectively NASH or NAFL, there are different treatment options. The main therapeutic goal is the prevention or at least deceleration of the disease progression, which means in case of steatosis prevention of NASH development, and – in case of steatohepatitis onset – to avoid progression of fibrosis and cirrhosis. However, the focus is not only the containment of the manifestations within the liver, but also the risk factors of the metabolic syndrome; such are insulin resistance and obesity. Following this paradigm, the modification of patients' lifestyle comes to the fore.

Weight loss, achieved by physical training or the change of food habits is usually changing the progression of fatty liver disease. A reduction of hepatic insulin resistance, adipose tissue and serum ALT is an often observed consequence[100]. Usually the elimination of unfavorable co-factors, e.g. alcohol or drugs with elevated hepatotoxicity, is also recommended.

If a reduction of weight cannot be achieved by the described measures or if the BMI of patients is already in a morbidly high range (>35-40) and the weight loss is urgent

due to severe illness, bariatric surgery is taken into consideration in an increasing number of cases. It has been shown, that in these patients the long term weight loss lead to a decreased level of hepatic fat accumulation. Analysis of bariatric patients showed also a decrease in mortality, morbidity and in detected cancers[101]. However, bariatric surgery is not a standard treatment of NAFLD patients yet, but the obtained achievements considering the reduction of steatosis, fibrosis as well as inflammation should be further investigated under the light of the prevention of NAFLD progression into cirrhosis[102].

In the last years, different approaches for drug based NAFLD treatments have been investigated. Metformin, one of the first line treatment options for pre-diabetics and T2DM patients, could not be proven to have any significant effect on steatosis. Neither histology nor transaminases showed an improvement compared to changes in lifestyle[19]. Pioglitazone, a PPAR-ɣ agonist showed positive effects by improving the status of inflammation and steatosis[103, 104]. For further evaluation, broad studies about long-term effects and the effect on fibrosis have to be conducted[14, 19, 105]. However, safety concerns regarding an increased risk of cardiovascular events remain and it is important to mention that these drugs are only licensed for diabetic patients.

Hepatic cell injury in NASH context is often referred to in the same line with oxidative stress. Hence, the impact of the antioxidant vitamin E is also in the focus of investigation for NASH treatment options. Randomized studies showed improvement

in inflammation and steatosis, whereas no effect on fibrosis could be proven[104]. Again, safety concerns regarding an increased risk of cancer prevail and clinical evidence only exists for non-diabetic patients.

An advanced degree of necroinflammation and fibrosis is often correlated with a deficient incidence of the secosteroid vitamin D in NAFLD patients[106]. The fact that it has been proven to play a relevant role in cancers as also in infectious and autoimmune diseases, where anti-inflammatory effects could be shown[107], makes it to a substance of interest in NASH therapy. It is currently investigated in further studies and remains a therapeutic opportunity with high potential[108].

1.3 MicroRNAs

Since microRNAs (miRNAs) have been discovered in 1993[109], scientists proofed a regulative function in various gene expression pathways.

These small RNAs of 19 to 23 nucleotides in total length regulate gene expression acting usually at posttranscriptional level. In majority (80%), their sequence is located in non-coding intronic sections of the genome[110, 111], where they can be found either as clusters of genes or as single genes, controlled by the promotor of the protein coding genes or by their own promotors[112]. They are involved in regulating the activity of thousands of genes and their protein synthesis[113]. MiRNAs are very stable in clinical samples as they are resistant to the degradation by ribonucleases. This is one reason why several miRNAs have been proposed as

attractive diagnostic tools for less invasive distinguishing of patient's health status[114], a fact, also bringing them in the focus of the study design of the work in hand. These miRNAs, usually evaluated in cell free plasma or serum are also called circulating miRNAs. The range of miRNA applications is getting broader as they are used in different clinical settings for early disease detection and monitoring of disease progression[97]; and - as they have been linked to a number of different diseases - also the first miRNA based therapy options are under investigation[115].

A microRNA specific database (http://www.mirbase.org) list over 28645 entries and associated publications and is steadily growing[116].

1.3.1 MiRNAs - An introduction

MicroRNAs are to date estimated to regulate more than 30% of the protein-coding genome in humans[117]. These small molecules are recognized today as key mediators in cellular processes, including cellular differentiation and -growth, proliferation as well as apoptosis[118, 119]. As shown in Figure 1-4, miRNAs are most commonly processed by RNA polymerase II, but also occasionally RNA polymerase III is responsible for transcription. The products are the long hairpin precursor structures called pri-miRNA[120, 121]. These precursors are poly-adenylated (3' end) and capped (5' end), just as other protein coding primary transcripts[122-124]. Their unique hairpin structure helps the microRNA specific biogenesis apparatus to separate them from other RNA stem loop-like structures in the nucleus[121]. The

processing pathway is canonical, where the pri-miRNA is going through a two-step mechanism which involves ribonuclease (RNase) III-family proteins Drosha and Dicer[125, 126]. This has the consequence that the newly synthesized pri-miRNA is cleaved by the nuclear Drosha, which forms, supported by its cofactor DGCR8 (DiGeorge syndrome chromosomal [or critical] region 8) the microprocessor complex for export from the nucleus by the nuclear export factor exportin 5 (Exp5)[127, 128]. MicroRNAs general function is acting as imperfect sequence guides to recruit a ribonucleoprotein (RNP) complex to the complementary RNA. By the usage of this paradigm, they act in a familiar manner to other RNP components, in particular by providing a binding component which is sequence-specific. This allows the RNP to act on a particular target[129]. This function is working with the same concept as messenger RNA (mRNA) splicing. Small nuclear RNAs are bound to corresponding proteins building a RNP (snRNP) which introduces splicing.[129, 130]

The miRNA-RNP complex is defined as RNA-induced silencing complex (RISC) and is using the miRNA to build a sequence specific complex with target mRNA. The complementary sequence is often between seven and eight bases. It is important to mention that longer stretches of complementarity might even enhance binding. The complementary sections of the miRNA and its target are preferentially located at the 5’ end of the miRNA, which is also called the seed (nucleotides 2-8 counted from 5’ end)[117]. If the complementarity at the 6-mer site is from nucleotides 2-7, it is usually not sufficient for the repression of the target mRNA, but an additional complement base on position 8 is enough for decreasing the expression of the target[131].

Altogether, there are five different classes of miRNA binding sites:

- Class I: Binding only relies on the seed
- Class II: Seed bind and complementarity in nucleotides 13-16
- Class III: Seed bind and complementarity in nucleotides 17-21
- Class IV: No seed binding, but resemble centered binding (nucleotides 9-16)
- Class V: Distributed or less stable binding

(Adapted from [133])

The RISC induced complex is often used in context with the term of gene silencing. This might give a wrong expression, as in most of the cases the result is not a total gene silencing, but more a regulating mechanism in the calibration of gene expression[129].

MiRNAs can direct the repression of target genes, which makes them not only conceptually, but also mechanistically related to RNA interference (RNAi) and small interfering RNA (siRNA). The mechanism of repression usually differs between different binding areas on the miRNA seed- and central region (nucleotides 2-12 of the miR). Generalized, if miRNA-target interaction consists of a high amount of complementarity the result is mRNA degradation; whereas with a decreasing amount of complementarity the function as regulating mechanism of gene translational repression is increasing. It is important to clarify, that miRNAs are not

working in a ‘black’ or ‘white’ mode by turning their targets totally on or off; they are fine tuning gene expression[113, 134].

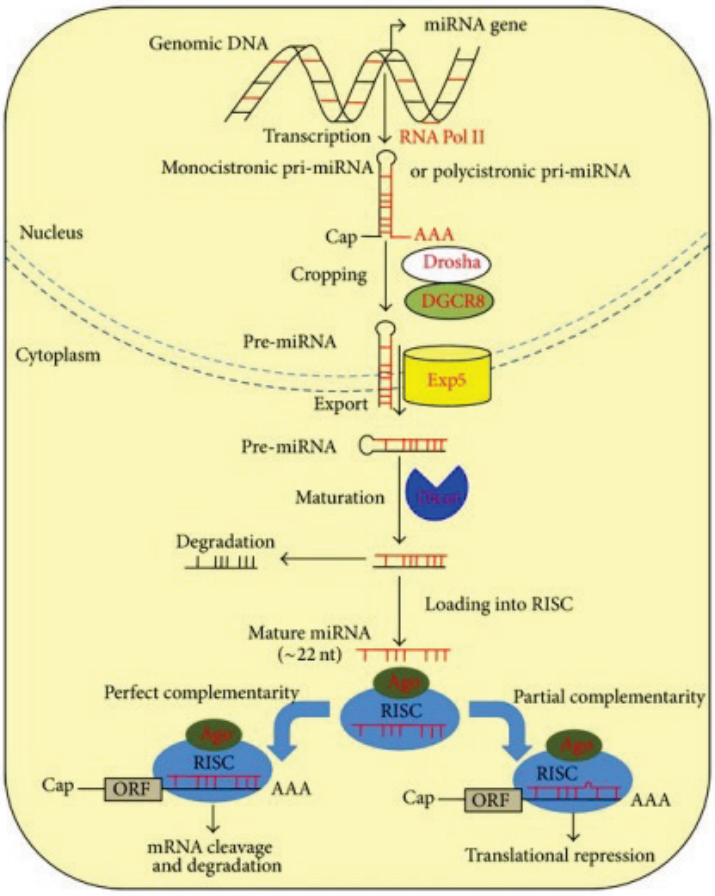

Figure 1-4: MicroRNA processing in nucleus and cytoplasm (reproduced from [132])

For microRNAs themselves, their appearance is also sometimes described to be *on* or *off*. But even if some miRNAs have very high temporal (e.g. let-7 in *Caenorhabditis elegans* development[135]) and local (e.g. miR-124 in the brain[136]) specificity, microRNAs are in general expressed in multiple cell and tissue types and

on different expression levels. The strict dichotomous model can be considered as exceptional cases[136].

The majority of miRNA effects are mediated by a decreased level of target mRNA, but also effects with an exclusive decrease of resulting protein expression are known, which can be caused by e.g. induced decapping or altered protein binding[137]. Bringing this observations in a practical context one can say that the observation of mRNA levels can be used to indicate miRNA targeting, but means also that the use of only mRNA profiling to determine miR targets may still miss relevant target genes, because the change of mRNA level might be small and not all targets show a decrease in expression[129].

An interesting fact about microRNAs is their ability of having opposing functions in different systems, which can illustrate the varying miRNA communications in different contexts. Given as an example, miR-125b can be downregulated in various cancers, such as hepatocellular, breast and lung. On the other hand, same miR-125b is associated to be upregulated in colorectal, pancreatic and gastric cancers as well as in some leukemias[138]. These findings show an oncogenic but also a tumor suppressive function of miR-125b, very specific to the corresponding environment. This particular difference is explained by the targets of miR-125b, such is p53. In above mentioned cancer tissues, where miR-125b is overexpressed, the result is a loss of p53 which blocks apoptosis. In other cancer tissues, p53 has a higher potential to be mutated and the downregulation of miR-125b leads to an expression

of oncogenic targets, such is epidermal growth factor receptor (EGFR) family members ERRB2/3 in breast cancer[138].

Also, even if microRNAs have been associated with single pathogenic outcomes they have very differing appearance patterns between tissue and serum. For instance liver-specific miR-122, which has been associated with downregulation in liver tissue in NAFLD in multiple studies[139], is showing a clear increase of appearance in corresponding individuals' serum[97]. As this miRNA has also been in the focus of investigation of the experimental part of the work in hand, both this observations could be validated. This can be explained by the fact that damaged hepatocytes release the miRNA into the blood[140-142], but gives an example about the complexity of interpreting the level of miRNA appearance.

1.3.2 MiRNAs in metabolic syndrome and liver disease

In the following, the key interacting miRNAs in relevant organs of the metabolic syndrome will be summarized, with more detailed information on their role in the context of NAFLD and rounded up with the emerging role of miRNAs in other pathogenic liver manifestations.

1.3.2.1 MiRNAs in the pancreas

The pancreatic islets consist of α- and β-cells. These play an important role in the context of metabolic body functions by producing and releasing insulin and glucagon; both hormones with a crucial role in carbohydrate and lipid metabolism in

other tissues. Changes in β-cells function and increased apoptosis can lead into a manifestation of T2DM. Recent studies showed the crucial interaction of miRNAs as mediators in the development of pancreatic β-cells but also in the induction of their dysfunction[143].

One of the first studies bringing miRNAs in the context of pancreatic β-cell development showed that the loss of the dicer protein lead to a reduction in β-cell mass as well as to an increase in development of hyperglycemia and T2DM[144, 145]. Further studies determined a crucial role for specific miRNAs in β-cell development, all started with miR-375, which showed a high increase in expression[146]. Also, the onset of hyperglycemia and impaired β-cell proliferation correlates with miR-375 silencing[147].

Several miRNAs, including miR-199, miR-383, miR-34a, miR-146a, miR-187, miR-15a, miR-30a, miR-30d, miR-96, miR-133, miR-148 and miR-182 have been shown to be involved in the progression of T2DM, pancreatic function, in the insulin synthesis regulation and secretion, and dysregulated levels of FFA and glucose[143, 148-157].

An important and highly conserved miRNA family in this context is miR-33. These miRNAs, with their information provided in the intronic sections of the *SREBP* genes in organisms ranging from drosophila to humans[158-161] show high impact on targets involved in the cholesterol export, such is e.g. the adenosine triphosphate binding cassette (ABC) transporters ABCA1 and ABCG1 and the endolysosomal transport

protein Niemann-Pick C1 (NPC1)[161]. Their crucial roles have been shown not only in the maintenance of cholesterol homeostasis, but also in regulation of fatty acid metabolism and insulin signaling[162]. MiR-33 has putative binding sites in the 3' untranslated region (3'UTR) of carnitine O-octaniltransferase (CROT), carnitine palmitoyltransferase 1A (CPT1a), hydroxyacyl-CoA-dehydrogenase (HADHB), AMP kinase subunit-α (AMPKα), and insulin receptor substrate 2 (IRS2) and is involved in gene expression of these genes, which has the direct consequence of a reduction of insulin signaling and fatty acid oxidation[162]. In summary miR-33a and –b in interaction with SREBPs are main regulators in the cholesterol metabolism, insulin signaling and fatty acid oxidation, which are major risk factors involved in the development of T2DM and the metabolic syndrome[162-165].

1.3.2.2 MiRNAs in white adipose tissue

The white adipose tissue (WAT) is the primary organ in the uptake and storage of excess circulating lipids. Therefore, WAT is a key player in the maintenance of metabolic function of the whole body and is involved in insulin sensitivity and appetite regulation by releasing signaling molecules, such as adiponectin and leptin[166, 167]. MetS associated obesity – characterized by increased accumulation of abdominal fat - is often involved in the development of hypertension, dyslipidemia and insulin resistance[2], which means that either excessive lipid accumulation or genetic abnormalities in WAT can also lead to various manifestations of the metabolic syndrome[168, 169].

The differentiation of pre-adipocytes into adipocytes, which are responsible for uptake and storage of circulating lipids and glucose, is mediated by a well investigated signaling cascade which involves the transcription factors PPARγ, SREBP-1 and C/EBPα/β (CCAAT/enhancer-binding protein alpha/beta).

	Involved miRNAs
Promotion of adipogenesis	miRNA cluster miR-17-92, miR-21, miR-26b, miR-30, miR-103, miR-143, miR-146b, miR-181, miR-204/211, miR-210, miR-375 and miR-637[173-185]
Impairing of adipocyte differentiation	let-7, miR-15a, miR-22, miR-27a/b, miR-130, miR-138, miR-155, mirR-193a/b, miR-205, miR-221, miR-222, miR-224, miR-344, miR-365, miR-369-5p and miR-448[186-201]

Table 1: MiRNAs involved in adipogenesis and adipocyte differentiation

Several microRNAs have been described to be involved in WAT differentiation (Table 1); also several of these displayed miRNAs show a difference in expression patterns in WAT between various conditions of obesity and the metabolic syndrome[170-172].

1.3.2.3 MiRNAs in the liver

MiRNAs have been proven to play a crucial role in liver metabolism, growth and regeneration as well as in disease.

The fact that miRNAs are key mediators in lipid metabolism, insulin signaling, cell growth and differentiation, apoptosis as well as inflammation leads to the conclusion, that miRNAs are playing a crucial role in the pathogenesis of the manifestation of the metabolic syndrome in the liver – into NAFLD.

An increasing number of studies are investigating different miRNA expression patterns related to the manifestation of NAFLD and the progression from bland steatosis to NASH. Among those, several already have been described and addressed above as key regulators of glucose-, cholesterol- and lipid-metabolism, which might lead to the conclusion, that hepatic deregulation of those miRNAs could cause intra-hepatic excessive accumulation of triglycerides and fatty acids[202].

The most abundant miRNA in the liver and also the first which could be correlated with lipid metabolism and homeostasis is miR-122[203]. The incidence of this miRNA is one of the main targets of the experimental part of this work. It has been investigated in liver tissue (see 4 Second manuscript) as well as in serum (see 3 First manuscript). In murine studies, miR-122 inhibition lead to a significant reduction of plasma cholesterol and increased levels of genes involved in hepatic cholesterol synthesis, e.g. 3-hydroxy-3-methylglutaryl-Coa synthase 1 (Hmgcs1), 3-hydroxy-3-methylglutaryl-Coa reductase (Hmgcr) and the 7-dehydrocholesterolreductase

(Dhcr7)[204]. In another murine study, a group could show how silencing of miR-122, based on antisense-nucleotides (antagomirs), increases the fatty acid β-oxidation pathway and decreases fatty acid and cholesterol biosynthesis[205].

Different animal models of NASH could also show other miRNA expression patterns which could be associated with the manifestation of the disease. Hepatic miRNA expression in a rat model, fed with hypercaloric diet enriched in fat (HFD) and fructose (HFD-HFr) showed an increase of miR-200a, miR-200b and miR-429 expression and a concomitant decrease in expression of miR-27a, miR-451 and again miR-122[206]. A C57BL/6J and DBA/2J mice model on a methyl-deficient diet, showed hepatic over-expression of miR-34a, miR-155, miR-200b and miR-221 whereas miR-29c, miR-122, miR-203 and miR-192 were significantly down regulated[207]. The latter was also part of the experimental design of the work in hand and will be further addressed later in this chapter. MiR-15b also showed characteristics of a key regulator of lipid metabolism in an animal model: Two studies, based on a rat HFD model and on the L02 hepatocyte cell line treated with FFA (palmitate) in culture, could show that in both models miR-15b was highly overexpressed, glucose consumption was reduced and triglyceride levels were altered. By taking these findings together, one could assume an increased risk of NAFLD onset, triggered by augmented miR-15b expression, which leads to an altered glucose and lipid metabolism[208].

A wide range of miRNAs where described in context with NAFLD pathogenesis. In an in vitro study, miR-10b lead to an increase of the triglyceride levels interacting with PPARα, which is involved in the catabolism of fatty acids[209] (1.2.2.). Other candidate miRNAs important to mention in this context are miR-33a and miR-33b which have been already described as an important miRNA family in pancreatic context (1.3.2.1). Those are located intronic within SREBF2 and SREBF1 genes and have positive influence on the expression of lipogenic as well as cholesterogenic genes[210]. Numerous studies have described miR-33a/b in close functional interaction with hepatic lipogenesis and cholesterol homeostasis[161, 162]. The earlier addressed (1.3.2.1) involvement in regulation of post-transcriptional repression of ABCA1 renders it a key mediator in the binding process from cholesterol to apolipoprotein A1 (APOA1) during HDL formation[211]. Furthermore, Goedeke et al. could describe a regulatory function of miR-33 family in SREBP target genes by interacting with the deacetylase NAD+ dependent sirtuin 6 (SIRT6) which has also influence on lipid and glucose homeostasis[212].

MiRNAs are not only connected to fatty liver disease, as their influence could also be shown on other entities of liver pathology. In the context of hepatitis B virus (HBV)-related liver disease, a panel of miRNAs - miR-602, miR-96, miR-29c and miR-148 - has been shown to be related to disease progression and virus infection[213-216]. These studies comprised detailed information about miRNA interactions in viral infections, including the fact that overexpression of miR-602 could be shown in the complete progression of HBV from early chronic hepatitis to HBV-related cirrhosis

and HCC; its crucial role in hepatocarcinogenesis is manifested by regulation of tumor suppressive gene Ras association domain family 1 isoform A (RASSF1A)[216].

In diseases related to hepatitis C virus (HCV) infection, miR-155 is playing an important role by promoting tumorigenesis and hepatocyte proliferation. The mechanism is based on Wnt signaling activation and therefore miR-155 has been suggested as useful biomarker for HCV related HCC[217].

This miR-155 has also been shown and described to be involved in innate and adaptive immune responses in the context of liver inflammation[218]. Liver inflammation in general can be caused by various contributing factors. Recently, miRNAs have been suggested to play a fine tuning role, triggering the intensity of the inflammatory response by interaction with central signaling molecules of liver inflammation. In this context eight miRNAs have been highlighted: miR-125b, miR-146a, miR-150, miR-181, let-7, miR-21, miR-155 and miR-132[219, 220].

It has been shown in the past that alcoholic steatohepatitis also leads to dysregulation of miRNA expression in the liver. A prominent example would be increased expression of miR-132[221], which has been shown to be a key player in inflammation in the past[219, 220, 222]. Alcohol consumption also leads to an elevated miR-212 level in the gut, which causes an inhibition of ZO-1 protein level, a tight junction protein, and is involved in alcohol-induced gut permeability[223]. In addition, miR-217 has been shown to be a key player in alcohol-induced steatosis since this miRNA is regulating SIRT1, an essential regulatory metabolic transcription factor[224].

It has been discussed under 1.2.4 that a crucial point in NAFLD diagnosis is the determination of alcohol consumption habits. Defining a miRNA expression pattern to discriminate AFLD from NAFLD patients would represent another important aim to shed new light in this difficult clinical differentiation.

These implementations at least give a hint towards the important role of microRNAs in the context of the metabolic syndrome and in the liver. The given examples should be a sufficient survey for the manifold facets of miRNA regulation in hepatic health and disease.

1.3.3 MiRNAs in circulating blood of NAFLD patients

MiRNAs have been shown to bear diagnostic and prognostic potential as markers in various cancer forms, such as breast-, ovarian-, lung-, prostate- and liver-cancer. Also, many other non-tumoral pathologies have been shown to be associated with miRNA expression in serum. Not even ten years ago, scientists proofed the presence of miRNAs is not intracellular- specific, as they could be detected in plasma, platelets, erythrocytes, and nucleated blood cells[225-227]. In blood plasma an enormous stability, resistant against numerous influences, could be shown[225, 227]. The following intensive search for the ‘protective factor’ avoiding their degradation brought interesting findings: the immense stability was caused by the fact, that miRNAs circulating in plasma and serum can be packaged in microparticles such as apoptotic bodies, exosomes or microvesicles[228, 229]. Another option to prevent their degradation is the association with RNA binding proteins, such as Argonaute2 (Ago2)[230] or even with lipoprotein complexes as HDL[231]. Finding miRNAs in

circulating microparticles suggested them to be involved in cell-cell-communication. This interesting field of investigation is gaining more and more importance; hence first results already have given a glance about the role of miRNAs in cell-cell interaction[228, 232]. First proven interactions of miRNAs to be involved in the pathophysiological progression of NAFLD in the liver have led into related studies in this field. Serum levels of miRNAs have been evaluated with the aim of describing potential non-invasive biomarkers for monitoring and diagnosis of NAFLD[233].

1.3.3.1 MiR-122, miR-21 and miR-192

The three miRNAs miR-122, -21 and -192 are of special interest within the experimental part this thesis (see 3 First manuscript). In the following part, their relevance will be discussed in various aspects.

In a study conducted by Yamada et al., miRNAs were evaluated in a Japanese study cohort with hepatic steatosis (diagnosed by ultrasound). The results showed increased levels of four miRNAs, all known to be implicated in cholesterol and fatty acid homeostasis. The different levels of miR-34a, miR-21, miR-451 and miR-122 showed differential expression comparing NAFLD patients and healthy controls[234]. These finding confirmed previous related studies, showing a correlation between altered miRNA expression and the pathogenesis of NAFLD. MiR-122 and -34a were shown before to have a significantly increased expression in serum of NASH patients, also positively correlated with their stage of inflammation and fibrosis[235].

Miyaaki et al. could demonstrate an altered level of miR-122 in patients' sera, correlating with progression of NAFLD and fibrotic liver damage[236]. The correlation could be explained by the fact, that persistent liver damage is causing a leakage from hepatocytes, which are the main source of miR-122. Damaged hepatocytes are releasing the miRNA into circulating blood leading to altered mir-122 levels[235]. Interestingly, Li et al. described the functional linkage between fibrotic liver damage and miR-122 which is mediated by an inhibitory effect on the stellate cell activation and collagen deposition within the liver[237].

A very recent study showed also an increased elevation of three miRNAs in NAFLD patients' serum; again miR-122, but also miR-192 and miR-375[97]. The findings presented in this particular study had also influence on the experimental design of the present study. One major strength of this study is the fact that the NAFLD diagnosis was based on liver biopsy, enabling a definite differentiation between bland steatosis and steatohepatitis. For the first time, a differential regulation of the three miRNA has been observed during the course of the disease from NAFL to NASH. The evaluation of their data detected for miR-122 a higher potential for NASH prediction compared to classic biomarkers as ALT, AST and CK-18 fragment levels. From these data and the fact, that miR-122 is highly expressed in the liver and shows a high rate of detection in serum, one can suggest that miR-122 has a high potential as non-invasive biomarker for NAFLD progression and fibrosis detection, which could be validated by the results shown in this thesis.

Before Pirola et al. presented their results mentioned above[97]; miR-192 has not been brought in context with NAFLD detection. The miRNA is known to be upregulated by the pro-fibrotic cytokine TGFβ1[238]. It also has been shown to share a pri-miRNA with miR-194. Besides the liver, miR-192 plays a crucial role in the kidney and in diabetic nephropathy[238]. This might uncover the bridge between different pathologies within the metabolic syndrome. MIR-192/-194 are both described to have a high expression in specific tissue including the liver[239].

One of the new findings that could be shown by the results of the study at hand is the differential expression level of miR-21 in patients' serum comparing NASH patients with NAFL patients and healthy controls (see chapter 3). An upregulation in NAFLD patients' sera has been shown before, but without discrimination between NAFL and NASH[234]. A possible reason could be that a classification of patients was mostly based on liver ultrasound only as discussed above.

As miR-21 was shown to be connected to more than one disease, interdisciplinary research to clarify its occurrence and function was undertaken and is still ongoing. A critical meta-analysis by Haider et al. is concerning miR-21 as biomarker in context of cardiac diseases and viral hepatitis[240]. Five studies showed an increase of miR-21 in context of cardiovascular disease[240]. In liver disease, respective miRNA has shown potential as a diagnostic biomarker for hepatitis B[241] and C[242] virus. Going one step further in those particular studies, miR-21 was also brought into the context of HCC in connection with viral hepatitis[243].

In the context of steatosis, the functional role of miR-21 was first explained via lycopene, an unsaturated carotenoid antioxidant, which can decrease hepatic steatosis by suppression of its downstream target fatty acid-binding protein 7 (FABP7) in mice[244]. As miR-21 was also one of the first oncomirs, a lot of its targets are known and described as tumour suppressor genes. Prominent examples would be acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A), phosphatase and tensin homolog (PTEN) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A member 4[245, 246].

An upregulation of miR-21 could be detected in hepatocytes after unsaturated fatty acid treatment, mediated by NF-κBp65/ mammalian target of rapamycin (mTOR)-complex. The determined mechanism was a specific 3'- UTR binding of miR-21 to the PTEN messenger transcript, initiating its degradation. With this important study the gap could be closed, explaining decreased PTEN expression in HFD treated rats but also in NAFLD patients, suffering from obesity[247].

The considerations of this knowledge about the multiple functions of the miRNA can be taken as an argument to use miR-21 in combination with other biomarkers has an even higher potential than using it exclusively.

1.3.3.2 Other miRNAs

As it has been mentioned in the previous section already, not only circulating miR-122, -192 and -21 have been set into context with NAFLD. Looking for a possible explanation for altered levels of miR-34a in serum[235], Hermeking et al. found miR-

34a regulative interaction with pro-apoptotic genes and tumor suppressor p53 transcription in NAFLD progression[248]. The elevated levels of miR-375[97] are in line with its role as key mediator of glucose homeostasis. Also, it is an essential part in adaptive β-cell expansion which is known to be one response to increasing insulin demand in insulin resistance[147].

The analysis of miRNA levels in serum leaves room for a wide spectrum of plausible studies for interconnections. Following this approach, a cohort study with NAFLD patients could show a correlated increase of blood glucose levels, BMI or ALT levels parallel with miR-15b serum level. These findings could also suggest miR-15b for a potential marker as potential diagnostic candidate for NAFLD[210], but could not bring more satisfying results until today.

The presented data is showing the high potential of miRNAs in circulation and their use as potential diagnostic biomarkers. The knowledge newly generated in this particular area will be addressed in detail in the manuscript shown in chapter 3.

1.3.4 MiRNAs in NAFLD liver tissue

The foregoing chapter has concerned the role of miRNAs in the context of NAFLD in circulating blood. In 1.2.2 it has been stated that the pathophysiology of NAFLD is not fully understood. Even if numerous studies have been undertaken within that area, several aspects of regulation and progression of the disease remain unclear.

First presented results have shown the potential role of miRNAs as key mediators in fatty liver disease[139, 235], but the full context of their interactions is still in the focus of scientific investigation. An exploratory approach investigating the differences of miRNA expression in NAFL and NASH patients and healthy controls will be discussed in *4, Second manuscript* within this thesis. This part of the experimental set-up was dedicated to explore miRNAs impacting the progression from simple steatosis to liver inflammation but also the initial manifestation of NAFLD. Evaluating the results, four miRNAs became targets of further investigations – miR-122, -21, -223 and -638. The following part is considering relevant knowledge about these candidates.

1.3.4.1 MiR-122, miR-21, miR-223 and miR-638

The role of miRNA 122 has been in the focus of this disquisition before. As the most abundant miRNA within the liver, it was also the first with a confirmed correlation to homeostasis and lipid metabolism[203]. The groundwork for this knowledge was laid by a study by Cheung et al.; their work showed a highly significant downregulation of miR-122 in NASH patients. The effects on its targets were functionally assessed by overexpression and silencing in an in vitro study. Several effects could be shown in the field of protein translation, cell proliferation, inflammation, apoptosis, oxidative stress and metabolism. The overexpression led to a downregulation of miR-122 targets whereas an antagomir based silencing caused a significantly increased protein level. These results suggested that lower levels of miR-122 stand in a direct relationship to an alteration of the lipid metabolism which is implicated in the

pathogenesis of NASH[139]. With this knowledge, several other studies could proof a relationship between the miR-122 and NAFLD. However, this miR is still in the focus of investigation as some targets and their regulating functions remain unexplored.

Some known facts about miR-21 have been already addressed in chapter 1.3.3.1 – it has an oncomir function and one of its known targets is PTEN. Still, the direct functional connection of miR-21 and hepatic steatosis remains unclear. As an upregulation of the miRNA in hepatic tissue could be shown[247] and could also be validated in the experimental setup of this work (see chapter 4), first approaches to circumvent this effect have been investigated. For example Seeger et al. recently published a study were they pharmacologically inhibited miR-21 on long-term in a rodent model of metabolic syndrome and obesity. The result was a significant reduction of body weight, serum triglycerides and adipocyte size[249]. Direct correlations to TGFβ-receptor 2 (TGFBR2)[249] as well as its demonstrated role in regulation of PPARα in the context of beta-oxidation[250] show the enormous potential of miR-21 as a key mediator of MetS and NAFLD and suggest it to remain in the focus of scientific investigation.

MiR-223 is a rather unexplored candidate in the context of human NAFLD. However, an altered hepatic expression in a rodent model of fatty liver disease could be observed[251], a fact that could also be validated in human liver tissue in the experimental part of this study (see chapter 4). The mouse model showed deregulation of hepatic iron homeostasis[251]. An in vitro study suggested recently that

miR-223 is involved in selective HDL cholesterol uptake by inhibition of hepatic scavenger receptor class B type I (SR-BI). This implies an important role of miRNAs in the modulation of cholesterol metabolism[252]. However, the full influence on NAFLD development and progression could not be clarified, yet. MiR-223 has also been investigated in the context of HCC where its contribution to the regulation of cell proliferation is suggested as it has been described in the context of colorectal cancer[253].

MiRNA 638 has not been brought into the context of metabolic manifestations before at all. Even in the extended context of liver diseases, there was no relevant data existing, until very recently Kumar et al. showed an antiviral potential of the miRNA in the context of HBV infections[254]. Other studies showed different regulations in colorectal cancer patients[255, 256] but are lacking a clarification of its functional pathways. A verification of predicted target genes, suggesting a regulatory role in the context of metabolic manifestations or a potential contribution to NAFLD development represent one of the desired goals addressed in this work.

1.4 Genes involved in NAFLD

In the experimental setup of the work in hand, not only the different expression patterns of miRNAs are in the focus of investigation, but also the expression of mRNAs in NAFLD patients and cultures cell lines is addressed. The aim of this part of the study was the detection of a direct interaction of altered miRNA levels on their

potential targets. This functional evaluation should bring new light in pathophysiological aspects of miRNAs in the context of NAFLD and should be the groundwork for subsequent functional analysis in an in vitro assay in cell culture. The obtained results will be discussed in detail under *4, Second manuscript.* In this context, three genes are in the focus of further investigations. In chapter 1.2.2, several genes, pathways and receptors standing in relation to the progression of NAFLD have been addressed. A detailed introduction of all relevant genes would go beyond the scope of this work; this is why the three prementioned genes of interest were selected for a more detailed treatise.

1.4.1 *FASN* and *JUN*

The gene of fatty acid synthase (*FASN*) is a potential target of three miRNAs in the focus of interest within this work – miR-122, -223 and -21.

FASN is often mentioned in the context of lipid homeostasis, a complex molecular mechanism, regulated by several receptors which are also known in the context of NAFLD, for instance LXR[257] and PXR[258, 259]. Fatty acid synthase (FAS) in one of the major lipogenic enzymes; and - as the liver is the main organ for lipogenesis - the expression level is constitutively high. Being regulated by SREBP1c[210] and directly associated with insulin resistance induced hepatic steatosis[260], FAS is suggested to be one of the targets of differentially expressed miR-122, -223 and -21 in NAFLD

patients. A possible alteration between the mRNA levels in NASH patients compared to NAFL patients would also be plausible, as the level of insulin resistance is also rising within this progression.

The proto-oncogene *JUN* is encoding for the C-Jun protein. C-Jun is subject of regulation by extracellular stimuli, such as oxidative stress and pro-inflammatory cytokines; factors which have also been associated with hepatic inflammation[261]. The *JUN* N-terminal kinases (JNKs) –pathway is relevant for *JUN* activation in cell proliferation and stress induced apoptosis[262]. In hepatocytes, C-Jun has been demonstrated to bear protective function from apoptosis, also suggesting the protein to have a protective function against apoptosis in HCC[263].

JUN transcripts are targets of the miRNA candidates of interest miR-21 and -223. The consideration of the gene as a relevant factor is based on the fact, that the JNK pathway is relevant for IRS-1/2 phosphorylation leading to insulin resistance[264, 265] and SREBP-1a phosphorylation[266]. The latter was a crucial factor in terms of obesity phenotype and fatty liver development in a rodent study. The transgenic animals lacking the SREBP-1a phosphorylation were prevented from the described manifestations[266].

1.4.2 PLIN5

The gene perilipin5 (*PLIN5*) is a member of the Perilipin family and is encoding for the lipid storage droplet protein5 (LSDP5). Members of this protein family are

essential for coating intracellular lipid storage droplets as well as their protection from lipolytic degradation[267]. The prevention from lipolysis is a crucial step in the development of hepatocellular steatosis. The expression of perilipins is highly abundant to adipocyte and steroidogenic cells, as for instance hepatocytes[268-270]. The function of perilipin is well investigated. The perilipin knockout in a mouse model showed a decrease in adipose tissue mass which was highly significant[271, 272]. The ability of the cells to respond to lipolytic stimuli disappeared and a continuously high level of lipolysis could be detected. Lipolysis is controlled by the phosphorylation state of perilipin[272-277]. A correlation in expression between perilipins and PPARs could be detected suggesting a regulative function[267]. All these facts already predispose *PLIN5* as a gene of interest in NAFLD development. Another reason would be the fact that it is one of the few predicted targets of selected miRNA of interest 638.

Following this, the potential interaction between *PLIN5*, miR-638 and the development of NAFLD was investigated in one part of the experimental setting of the work in hand and will be described in detail in chapter *4, Second manuscript.*

2 OBJECTIVES

2.1 Aim of the project and addressed topics

NAFLD with its two different manifestations NAFL and NASH is an increasing health problem in western countries. Whereas patients suffering from simple steatosis have no influence on mortality, NASH patients have an increased risk to develop severe liver disease, such as fibrosis, cirrhosis and HCC. The pathophysiology of the development of NAFLD is still not entirely clear, as well is the search for stimuli, triggering or regulating the progression from NAFL to NASH still ongoing. Considering the diagnosis of NAFLD, the gold standard to differentiate between NAFL and NASH is still liver biopsy with histological examination, as no valid biomarker has been described yet. Within this work, these two fields of interest were considered. On the one hand, miRNAs expression in NAFLD patients' serum and healthy controls was detected and evaluated, aiming to define an expression pattern valid as a NASH biomarker. On the other hand, miRNA expression patterns were investigated in tissue obtained from human liver biopsies. The aim was to find differentially expressed miRNAs responsible for the manifestation or the progression of the disease. Potential candidates were further addressed in functional studies in cell culture and correlated with obtained data from messenger RNA expression studies.

Within this work the following topics were addressed:

- Evaluation of miRNA expression patterns in serum as well as design of a simplified scoring system in combination with the apoptosis marker CK-18 fragment level to evaluate its prognostic potential as NASH biomarker.
- Contribution of four miRNAs in human liver tissue to the development and progression of NAFLD, followed by functional studies to evaluate the pathophysiological mechanisms.

When this project was started some relevant miRNAs in the context of NAFLD were already defined, however, data from human liver tissue were lacking or a clear differentiation between NAFL and NASH was not obtained. During the time of this work more and more evidence arose that miRNAs play a crucial role as well in NAFLD pathophysiology as also in the context of NAFLD diagnosis. It could also be shown that data from serum and liver tissue are rarely subject of comparable expression. We could confirm earlier results obtained by other groups with our data. Our study was aiming to identify functionally relevant miRNAs in liver tissue as mediators of central signaling pathways and clinically relevant pathophysiological events in fatty liver disease. The data obtained in the serum study were used for the detection of the role of certain miRNAs as potential prognostic biomarkers to monitor the progression of fatty liver disease.

2.2 Contribution to further projects during the time of the dissertation

(Not addressed in detail within this work)

Impact of genetic SLC28 transporter and ITPA variants on ribavirin serum level, hemoglobin drop and therapeutic response in patients with HCV infection

Various independent studies discuss a potential impact of genetic SLC28 transporter and inosinetriphosphatase (ITPA) variants on the serum level of ribavirin, the hemoglobin drop and the influence on the therapeutic outcome in patients suffering from a hepatitis C virus (HCV) infection. A cohort study assessed SNP – associations with their impact on HCV treatment.

Publication

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3 FIRST MANUSCRIPT

Performance of serum microRNAs -122, -192 and -21 as biomarkers in patients with non-alcoholic steatohepatitis

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Author contribution:

- Serum sample collection (MR, HB, AG)
- RNA isolation (PB)
- Quantitative real time PCR, analysis, interpretation (PB, AG)
- Statistical Set-Up (CH, CM)
- Statistical verification (CM)
- Design and coordination of the study (PB, JS, BM, AG)
- Manuscript writing (PB, AG, CM)
- Figure design and arrangement (PB, AG)

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RESEARCH ARTICLE

Performance of Serum microRNAs -122, -192 and -21 as Biomarkers in Patients with Non-Alcoholic Steatohepatitis

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-

Abstract

Objectives

Liver biopsies are the current gold standard in non-alcoholic steatohepatitis (NASH) diagnosis. Their invasive nature, however, still carries an increased risk for patients' health. The development of non-invasive diagnostic tools to differentiate between bland steatosis (NAFL) and NASH remains crucial. The aim of this study is the evaluation of investigated circulating microRNAs in combination with new targets in order to optimize the discrimination of NASH patients by non-invasive serum biomarkers.

Methods

Serum profiles of four microRNAs were evaluated in two cohorts consisting of 137 NAFLD patients and 61 healthy controls. In a binary logistic regression model microRNAs of relevance were detected. Correlation of microRNA appearance with known biomarkers like ALT and CK18-Asp396 was evaluated. A simplified scoring model was developed, combining the levels of microRNA in circulation and CK18-Asp396 fragments. Receiver operating characteristics were used to evaluate the potential of discriminating NASH.

Results

The new finding of our study is the different profile of circulating miR-21 in NASH patients ($p<0.0001$). Also, it validates recently published results of miR-122 and miR-192 to be differentially regulated in NAFL and NASH. Combined microRNA expression profiles with CK18-Asp396 fragment level scoring model had a higher potential of NASH prediction compared to other risk biomarkers (AUROC = 0.83, 95% CI = 0.754–0.908; $p<0.001$). Evaluation of score model for NAFL (Score = 0) and NASH (Score = 4) had shown high rates of sensitivity (91%) and specificity (83%).

alcoholic steatohepatitis; NAS, NAFLD activity score; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK18-Asp396, cytokeratin-18 fragments M30; IL-1RA, interleukin 1 receptor antagonist; miRNA/ miR, microRNA; HC, healthy controls; MOC, moderate obese cohort; SOC, severely obese cohort; ELISA, enzyme-linked immunosorbent assay; RT-q-PCR, real-time quantitative PCR; SEM, standard error of mean; ROC, receiver operating characteristic; AUROC, area under ROC; OR, odds ratio; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; *SLC7A1*, solute carrier family 7, member 1 gene; TGFβ1, transforming growth factor beta 1; PTEN, phosphatase and tensin homolog.

Conclusions

Our study defines candidates for a combined model of miRNAs and CK18-Asp396 levels relevant as a promising expansion for diagnosis and in turn treatment of NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in developed countries with a prevalence of 20 to 35% [1, 2]. Non-alcoholic fatty liver (NAFL) is defined as bland steatosis and must be distinguished from non-alcoholic steatohepatitis (NASH) which is characterized by inflammatory infiltrates, ballooning and necroapoptosis. 5% to 20% of NAFLD patients develop a progression from NAFL to NASH. This is of particular importance since the presence of NASH and/or advanced fibrosis has been associated with an increased overall mortality in these patients [3, 4]. Liver biopsy with subsequent histological examination still remains the gold standard in clinical practice to distinguish NAFL from NASH. A common scoring system to differentiate between NAFL and NASH is the NAFLD activity score (NAS) which is defined by the sum score of steatosis, ballooning, and lobular inflammation [5].

For optimal follow-up and treatment of NAFLD patients, development of non-invasive diagnostic tools to differentiate between NAFL and NASH remains crucial. In clinical practice, clinical presentation including age, BMI, presence of type 2 diabetes and routine parameters such as AST and ALT predict liver-related events [6–8]. Based on these observations the NAFLD risk score has been developed [9]. Cytokeratin-18 (CK18-Asp396), an intermediary filament protein proteolytically cleaved by caspases during liver cell apoptosis [10, 11]. The resultant CK18-Asp396 fragments have been investigated as a liver specific apoptosis and NASH biomarker [12]. However, the sensitivity and positive predictive value for NASH is low [13] and current practice guideline do not recommended CK18-Asp396 fragments as a single marker for the detection of NASH [14]. Reliable serum biomarkers are still not available and further promising candidates such as the interleukin 1 receptor antagonist (IL-1RA) have been investigated in NASH patients [15].

Since microRNAs (miRNAs) have been discovered, scientists demonstrated a regulative function in various gene expression pathways. MiRNAs are very stable in clinical samples as they are resistant to the degradation by ribonucleases. This is one reason why circulating miRNAs have been proposed as attractive diagnostic tools for non-invasive assessment of a pathological state of their origin organ/ tissue from peripheral blood [16]. The range of miRNA applications is getting broader as they are used in different clinical settings for early disease detection and monitoring of disease progression [17]. Very recently, Pirola et al. made an attractive approach to improve the non-invasive assessment of NAFLD by using miRNAs as potential diagnostic NASH markers [17].

Given the vast variability of miRNA results in different studies assessing the same disease entity and important methodological differences between these studies [18–21], validation of these results together with the evaluation of additional miRNA candidates is warranted. We hypothesize that miRNAs in circulation as molecular marker of cell damage would indicate the presence of NASH and contribute in combination to routine serum markers. The aim of this study is to establish potential diagnostic miRNAs as biomarkers for the differentiation between NAFL and NASH by defining a specific single or a grouped expression pattern for non-invasive NASH diagnosis.

Material and Methods

Study design, patients and healthy controls

A total of 137 NAFLD patients >18 years and 61 healthy controls (HC) were enrolled between July 2007 and August 2014. NAFLD patients consisted of two different cohorts of patients, one with a majority of moderate obesity from Hannover Medical School (MOC; n = 81; 16 NAFL/ 65 NASH) and one with a majority of severely obese patients mostly undergoing bariatric surgery from Würzburg University Hospital (SOC; n = 56; 34 NAFL/ 22 NASH). Blood samples were collected prior to any therapeutic procedure. Table 1 shows baseline parameters of the

Table 1. Baseline patient characteristics of study cohorts.

'Severely obese cohort' (SOC)			
Total: N = 99 (%)	HC: N = 43 (43%)	NAFL: N = 34 (34%)	NASH: N = 22 (23%)
Sex, N (%): Male; Female	11 (26%); 32 (74%)	8 (24%); 26 (76%)	7 (32%); 15 (68%)
Age: Mean ± sd; Median (quartiles)	26.5 ± 3.2; 26 (21–37)	45.4 ± 10.6; 44.5 (22–64)	50.9 ± 10.8; 53 (30–77)
BMI: Mean ± sd; Median (quartiles)	21.4 ± 2.5; 21 (17.5–26.3)	49.4 ± 7.2; 49.7 (37.6–69.8)	48.7 ± 9.3; 47 (37–66.2)
Fasting glucose [mg/dl]:			
Mean ± sd; Median (quartiles)	n.d.	102 ± 24.5; 97 (71–178)	117.5 ± 51.5; 93.5 (71–244)
Triglycerides [mg/dl]: Mean ± sd; Median (quartiles)	n.d.	166.7 ± 98.1; 143 (47–531)	188.2 ± 74.9; 162 (90–369)
Cholesterol total [mg/dl]: Mean ± sd; Median (quartiles)	n.d.	197.7 ± 47.3;195 (114–307)	195.2 ± 28.7; 195 (128–258)
AST [U/l]: Mean ± sd; Median (quartiles)	20.4 ± 6.1; 19 (11.6–41.4)	30.8 ± 15.5; 25.5 (13.2–85.9)	45.9 ± 26.7; 36.3 (14–119
ALT [U/l]: Mean ± sd; Median (quartiles)	18.7 ± 8.0; 15.8 (10–46.6)	32.8 ± 18.2; 27 (13–179.4)	50.6 ± 20.3; 53.4 (19–89)
CK18-Asp396 [U/l]: Mean ± sd; Median (quartiles)	170.6 ± 54.8; 168 (91–291)	235.8 ± 118.2; 218 (111–646)	448.1 ± 252.4; 345 (139–996)
NAS: N = n.d./0/1-2/3/≥4; (%)	43/0/0/0/0; (100/0/0/0/0)	4/1/12/11/6; (12/3/35/32/18)	1/0/0/0/21; (5/0/0/0/95)
Fibroscan Elasticity [kPa]: Mean ± sd; Median (quartiles)	4.3 ± 1.1; 4.1 (2.5–7.1)	9.3 ± 5.3; 8.1 (3.5–25.4)	13.1 ± 10.5; 10.4 (3.6–47.2)
Fibrosis: N = n.d./0/1/2/3/4; (%)	43/0/0/0/0/0; (100/0/0/0/0/0)	6/15/11/0/0/2; (18/44/32/0/0/6)	2/7/5/3/2/3; (9/32/22/14/9/14)
'Moderate obese cohort' (MOC)			
Total: N = 99 (%)	HC: N = 18 (18%)	NAFL: N = 16 (16%)	NASH: N = 65 (66%)
Sex, N (%): Male; Female	6 (33%); 12 (67%)	12 (75%); 4 (25%)	39 (60%); 26 (40%)
Age: Mean ± sd; Median (quartiles)	33.4 ± 9.3; 31 (24–57)	45.3 ± 10.5; 46 (21–63)	47.2 ± 11.7; 48 (19–71)
BMI: Mean ± sd; Median (quartiles)	n.d.	31.6 ± 6.3; 31 (25–48.1)	30.6 ± 4.1; 30 (25.2–45.9)
Fasting glucose [mmol/l]: Mean ± sd; Median (quartiles)	n.d.	6.2 ± 1.8; 6 (4.6–10.7)	6.1 ± 1.5; 6 (3.9–9.6)
Triglycerides [mg/dl]: Mean ± sd; Median (quartiles)	n.d.	157.2 ± 112.0; 108 (61–502)	189.9 ± 124.9; 151 (64–736)
Cholesterol total [mmol/l]: Mean ± sd; Median (quartiles)	n.d.	209 ± 30.4; 206 (143–259)	222.1 ± 56.4; 213 (143–403)
AST [U/l]: Mean ± sd; Median (quartiles)	n.d.	43.8 ± 19.0; 38 (23–96)	49.7 ± 20.7; 44 (16–116)
ALT [U/l]: Mean ± sd; Median (quartiles)	n.d.	66.9 ± 43.5; 60 (26–176)	82.2 ± 47.0; 73 (13–221)
CK18-Asp396 [U/l]: Mean ± sd; Median (quartiles)	125.2 ± 25.3; 124 (85–185)	183.8 ± 86.8; 149 (79–429)	380.7 ± 318.1; 260 (78–1897)
NAS: N = n.d./0/1-2/3/≥4; (%)	18/0/0/0/0; (100/0/0/0/0))	0/0/16/0/0; (0/0/100/0/0)	0/0/0/17/48; (0/0/0/26/74)
Fibroscan Elasticity [kPa]: Mean ± sd; Median (quartiles)	n.d.	5.7 ± 1.4; 5 (3.3–9.1)	8.2 ± 4.5; 7 (3.7–25.7)
Fibrosis: N = n.d./0/1/2/3/4; (%)	18/0/0/0/0/0; (100/0/0/0/0/0)	2/8/6/0/0/0; (12/50/38/0/0/0)	3/21/26/10/4/1; (5/32/40/15/6/2))

Results are expressed as mean ± standard deviation, as well as median (quartiles) unless indicated differently.

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moderate obese and severely obese cohort as well as controls. Patients with significant alcohol consumption (females > 20g/day and males > 30g/day) and any pre-existing liver disease were excluded. Healthy control subjects seeking a routine health check-up at the Würzburg University Hospital or Hannover Medical School had no evidence or history of liver pathology, unremarkable liver ultrasound, normal liver stiffness and no elevation of serum transaminases AST and ALT. Furthermore, history of coronary heart disease, hypertension, valvular disease, any arrhythmia or systemic disease resulted in exclusion of controls from the study.

Routine clinical and laboratory parameters were determined at baseline (age, BMI, fasting glucose, triglyceride, total cholesterol, AST, ALT, CK18-Asp396) and liver stiffness was measured by transient elastography (Fibroscan®). For all patients, liver biopsies have been obtained either percutaneously or during bariatric surgery (frustule size >20 mm; min.10 portal tracts); histology has been evaluated by an experienced pathologist, who was blinded to the results of Fibroscan and defined the presence of NASH and the NAS score [5]. The study was approved by the Ethics Committee of Hannover Medical School, the Ethics Committee of Würzburg University, and the Zürich Cantonal Ethics Committee. Written informed consent was obtained from each participant and the entire study was conducted in accordance with the declaration of Helsinki.

CK18-Asp396 measurement

The apoptosis-associated neoepitope CK18-Asp396 was measured by using the M30-Apoptosense ELISA according to the manufacturer's instructions (Peviva, Bromma, Sweden) and as described previously [22].

Blood samples and RNA isolation

Small RNA fraction was extracted from 200 μL serum using the miRCURY TM RNA isolation kit—Biofluids (Exiqon A/S, Denmark) according to the manufacturer's protocol (v1.4; April 2014) with three minor variations. Specifically, before starting with isolation, 1μg of MS2 RNA from bacteriophage MS2 (Roche, Switzerland) was added to stabilize RNA during cDNA synthesis. Additional, 20 mg glycogen, RNA grade (Thermo Scientific) was added to inert co-precipitant of nucleic acids. The Exiqon UniSP6 Spike-in-Kit was added to monitor the yield of RNA isolation via quantitative real-time PCR (RT-q-PCR). RNA was eluted from spin columns in 50 μL nuclease-free water.

cDNA-synthesis and RT-q-PCR

RNA (2 μL) was reverse transcribed in 20 μL reactions using the miRCURY LNA Universal-RT miRNA PCR kit (Exiqon A/S). The cDNA was diluted 1:40 and amplified in 10 μL PCR reactions using specific LNA primer sets and the ExiLENT SYBR Green Master Mix (Exiqon-A/S). The amplification was carried out on a 7900HT real-time PCR instrument (Applied Biosystems). As control, RT-q-PCR for miR-425 [23, 24] and miR-103 [25–27] (as reference miRNAs/ global mean [28–30]), miR-16 (as a reference to avoid the bias of relevant hemolysis) [31, 32] and UniSp6 (as monitor of efficiency of reverse transcription as recommended by providing company Exiqon for cDNA transcription [28]) were performed to guarantee equal quality standard.

Statistics

Statistical analyses were performed with SPSS (Version 22, IBM, NY) and graphs were created with Prism5 (GraphPad Software, La Jolla, CA).

P-values < 0.05 were considered statistically significant ($^{*}< 0.05$, $^{**}< 0.01$, $^{***}< 0.001$, $^{****}< 0.0001$). Quantitative data were expressed as mean ± standard deviation unless otherwise indicated. For relative miRNA expression average expression of miR-103 and miR-425 was considered for normalization; $2^{-\Delta}$ Ct–method was used. To compare miRNA expression, Mann-Whitney U test was applied. For binary logistic regression with SPSS in combined group (SOC and MOC) the origin of the sample was included as co-variable to correct for differences between the subgroups. Correlation between variables was calculated using Spearman's Rank correlation test. Scoring of miRNA expression was performed by separating their expression in the entire NAFLD group by median (S1 Table), giving a '0' for lower and equal values (lower risk) and a '1' for higher values (higher risk), which was chosen to avoid bias regarding different RNA concentrations. Sum scores were in a range between 0 and 3 (or 0 and 4 when including CK18-Asp396 as a fourth parameter).

For characteristic factors in combined patient group (SOC and MOC), receiver operating characteristic (ROC) curve was calculated and area under ROC (AUROC) was evaluated with SPSS.

Correction for multiple testing was not applied, as the focus of the study was rather exploratory for the use of miRNAs as diagnostic biomarkers.

Results

Differential expression of circulating miRNAs

Four different miRNAs (miR-122, miR-192, miR-21, miR-223) which have been involved in chronic inflammatory liver disease and particularly NAFLD [17, 19, 33, 34] were quantified in patient sera. The evaluation of miR-122 in both cohorts showed significant expression profile change between control group and simple steatosis group ($p<0.0001$) and a significant further increase between NAFL and NASH ($p<0.0001$) (Fig 1). The difference between HC and NAFL was significant in both cohorts (moderately obese (MOC) and severely obese (SOC) patients), whereas the difference between NAFL and NASH was only significant in SOC ($p = 0.0051$). In MOC, in spite of consistent direction of effects, statistical significance was not obtained (S1 and S2 Figs). For miR-192 the same pattern of detected miRNAs as for miR-122 has been observed with a significant increase in NAFL and NASH compared to HC (Fig 1). Again, the difference between NAFL and NASH was only significant in SOC ($p = 0.0022$) (S1 and S2 Figs). Although the absolute difference was small, a significant increase of miR-21 expression was observed for the NASH group compared to both other groups ($p<0.0001$) and could be detected for both cohorts in separate (Fig 1, S1 and S2 Figs) ($p(SOC) = 0.0007$; $p(MOC) = 0.0075$). No relevant change in miR-21 profile between controls and simple steatosis could be observed in both cohorts. An increase of miR-223 expression profile in serum of NASH patients compared to NAFL and HC was only detectable in the severely obese cohort ($p = 0.0338$), but was absent in MOC (Fig 1, S1 and S2 Figs).

To compare miRNA profile level with histological scores, delta Ct measures were correlated to the corresponding NAS. For three miRNAs 122, 192 and 21 a significant discrimination for groups with NAS n.d. + 0–2, 3+4 and 5+ has been detected with an overall trend towards lower delta Ct measures with increasing NAS (Fig 2).

Contribution of miRNA and baseline patient parameters to the presence of NASH

In the following the statistical contribution of miRNA serum levels to the diagnosis of NASH was investigated in relation to established baseline factors by applying binary logistic

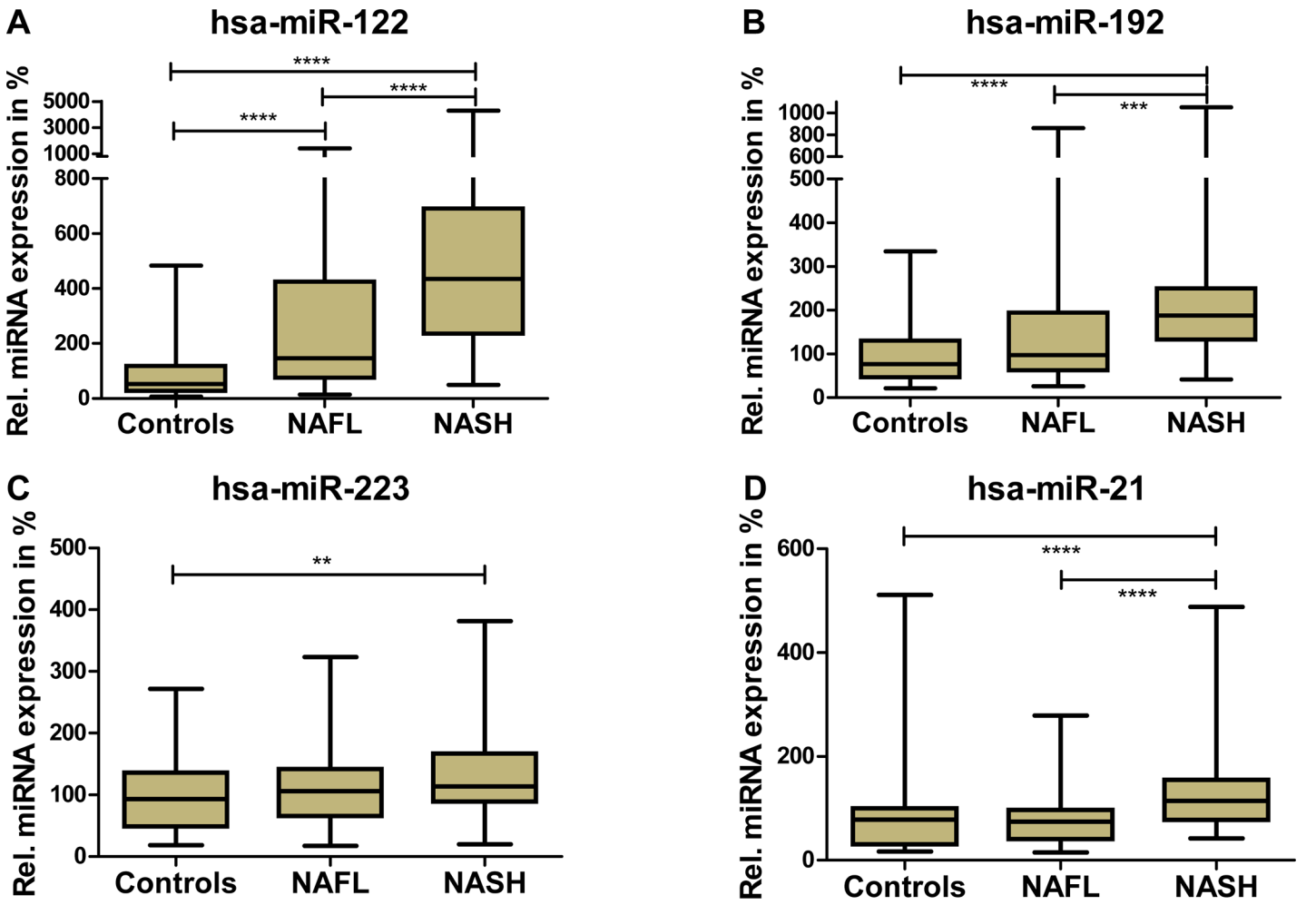

Fig 1. Relative detection profile of four investigated circulating microRNAs. Pooled data from both study cohorts (SOC & MOC). Displayed microRNAs show significant difference in detection between NASH patients and healthy controls. MicroRNAs -122 (A), -192 (B) and -21 (D) discriminate between bland steatosis and NASH patients. For miR-223, this difference could only be detected in one cohort (SOC; S1 Fig). MicroRNA data is normalized with miR-103 and miR-425. (Displayed: Median + interquartiles-range; whiskers = quartiles).

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regression. Established predictive parameters such as age, sex and BMI were considered as candidate co-variables for adjustment if a significant difference between the groups was calculated. However, none of those baseline parameters had a significant influence on the presence of NASH in both cohorts combined and in separate (Table 2).

In the combined analysis of both cohorts the deviation in size between the subgroups was taken into consideration for correct adjusting. After adjustment for origin the three miRNAs 122, 192 and 21 turned out to be highly significant co-variables in the logistic regression analysis (OR 0.592/p = 0.001, OR 0.487/p = 0.002 and OR 0.309/p<0.0001, respectively) (Table 2). In MOC patients, parameters of significant influence were miR-21 and the cellular apoptosis-marker CK18-Asp396 ($p \leq 0.05$). In SOC patients, the number of relevant parameters further included miR-122 and miR-192, whereas CK18-Asp396 was not significant in this subgroup. From these results, it appeared straightforward to combine expression of miRNA-122, -192 and -21 for further analysis with the aim of defining a combined score of individual miRNA markers for non-invasive NASH diagnosis.

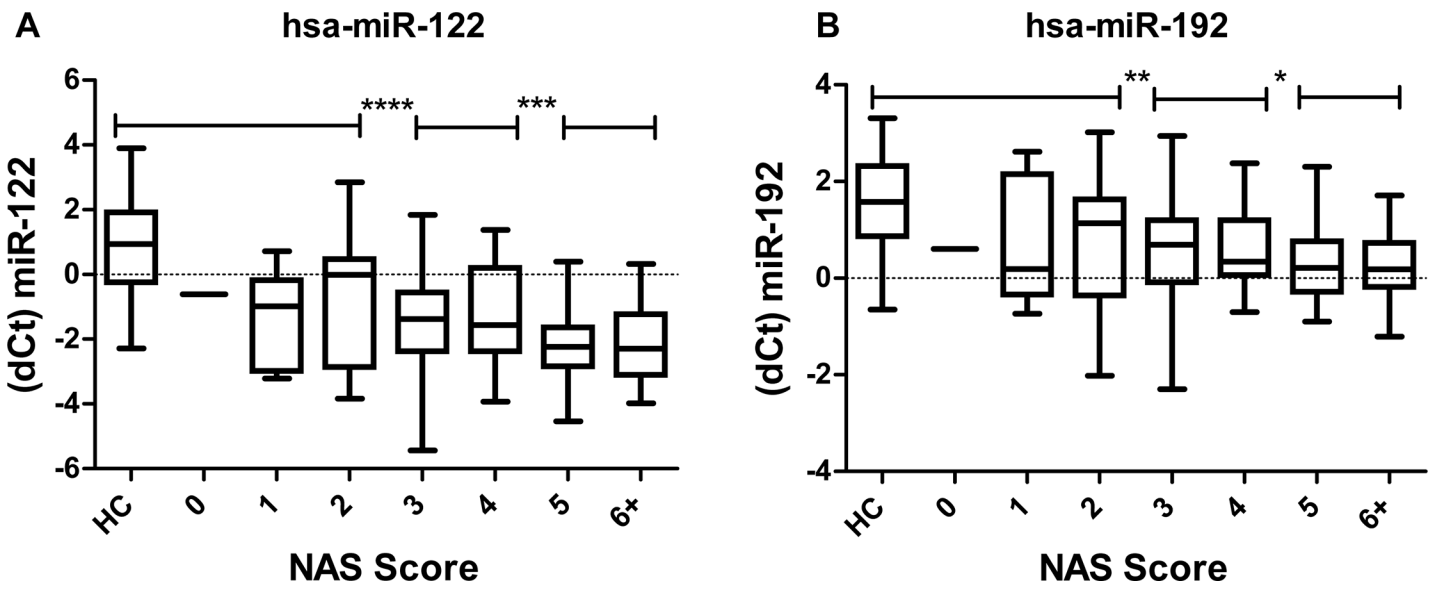

Fig 2. Circulating MicroRNA profile in comparison to histological NAS score. MicroRNA detection profile is shown as delta Ct. Pooled patient groups with NAS n.d.+ 0–2, 3+4 and 5+ could be significantly discriminated by lower delta Ct measures for miR-122 (A), miR-192 (B) and miR-21 (data not shown; p = 0.025–0.0258).

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OR is <1 despite of a higher expected risk, as it is calculated with Δ-Ct values which have an inverse correlation to increasing miRNA counts.

Correlation of routine serum parameters and miRNA expression profiles in circulation

In a next step detected miRNA profiles were correlated to available routine serum parameters such as ALT and CK18-Asp396 fragments (Fig 3). As miR-122 and miR-192 showed a very similar profile pattern in the different patient groups, their correlation was highly significant (R = 0.83, p< 0.0001, Fig 3). Both had a significant and positive correlation with serum ALT (miR-122: R = 0.53, p< 0.0001; miR-192: R = 0.45, p< 0.0001) (Fig 3). CK18-Asp396 fragment levels increased with the development of bland steatosis and the progression to NASH (Fig 3, S3 Fig). Again, a positive and significant correlation of CK18-Asp396 fragments to respective miRNA levels has been observed (miR-122: R = 0.48, p< 0.0001; miR-192: R = 0.36, p< 0.0001) (Fig 3). For miR-21 a positive significant correlation could be shown to ALT level but no correlation to CK18-Asp396 or other miRNAs could be determined (S1 Table). For miRs-21, -122 and -192 profile levels were also correlated with single NAS parameters (degree of steatosis, ballooning, lobular inflammation and fibrosis). A relevant correlation of all individual miR levels could be detected in comparison to the degree of steatosis. Also, a significant correlation of lobular inflammation to miR-21 and miR-122 could be found (S1 Table).

Diagnostic performance of routine serum parameters, CK18-Asp396 fragments and miRNA levels in circulation

As a final step, diagnostic performance of miR-122, -192 and -21 in a combination score compared to routine parameters such as ALT and CK18-Asp396 fragments has been analyzed in NAFLD patients.

A simplified scoring system has been developed dividing measured expression in both patient groups by the median (applied cut-offs shown in S2 Table) and subsequently scoring a

Table 2. Binary logistic regression analysis.

SOC			
	OR	**Confidence Interval (95%)**	**p-value**
Age	1.049	0.995–1.106	0.076
Gender	1.517	0.458–5.020	0.495
BMI	0.995	0.935–1.060	0.887
CK18-Asp396	1.001	0.999–1.002	0.421
miR-122	0.526	0.332–0.834	0.006 **
miR-192	0.416	0.216–0.801	0.009 **
miR-223	0.556	0.306–1.010	0.054
miR-21	0.313	0.148–0.660	0.002 **
MOC			
	OR	**Confidence Interval (95%)**	**p-value**
Age	1.014	0.968–1.063	0.552
Gender	0.5	0.145–1.72	0.272
BMI	0.956	0.842–1.085	0.487
CK18-Asp396	1.008	1.001–1.014	0.021 *
miR-122	0.666	0.430–1.032	0.069
miR-192	0.584	0.299–1.143	0.117
miR-223	0.858	0.382–1.927	0.71
miR-21	0.302	0.112–0.814	0.018 *
SOC and MOC (adjusted for origin)			
	OR	**Confidence Interval (95%)**	**p-value**
Age	1.03	0.995–1.066	0.093
Gender	0.863	0.374–1.992	0.73
BMI	0.988	0.933–1.046	0.674
CK18-Asp396	1.002	1.000–1.003	0.09
miR-122	0.592	0.434–0.808	0.001 ***
miR-192	0.487	0.308–0.770	0.002 **
miR-223	0.643	0.402–1.029	0.066
miR-21	0.309	0.170–0.561	0.000 ****

*: 0,01 < p ≤ 0,05
**: 0,001 < p ≤ 0,01
***: 0,0001 < p ≤ 0,001
****: p ≤ 0,0001

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lower or equal appearance with '0' and a higher with '1' finally summing up the results for all three miRNAs to a total score from 0 to 3. Alternatively, a combination with CK18-Asp396 fragment levels was applied, scored by the same method as miRNA expression with a total score from 0 to 4. To evaluate the diagnostic performance of this miRNA sum scoring, we performed a multivariate ROC and AUROC and compared it to ALT and CK18-Asp396 fragments alone (Fig 4).

Prediction with combined miRNA profiles showed an increase in AUROC compared to ALT alone (AUROC = 0.77) with the same diagnostic performance as CK18-Asp396 fragments (both AUROC = 0.81). Combination of these two predictors by applying a scoring system from 0–4 further increased the area under ROC by additional 0.02 (= ΔAUROC), to a value of 0.83 (Fig 4), which is showing a high sensitivity for the absence of steatohepatitis (score 0) and the diagnosis of NASH (score 4) (S4 Fig).

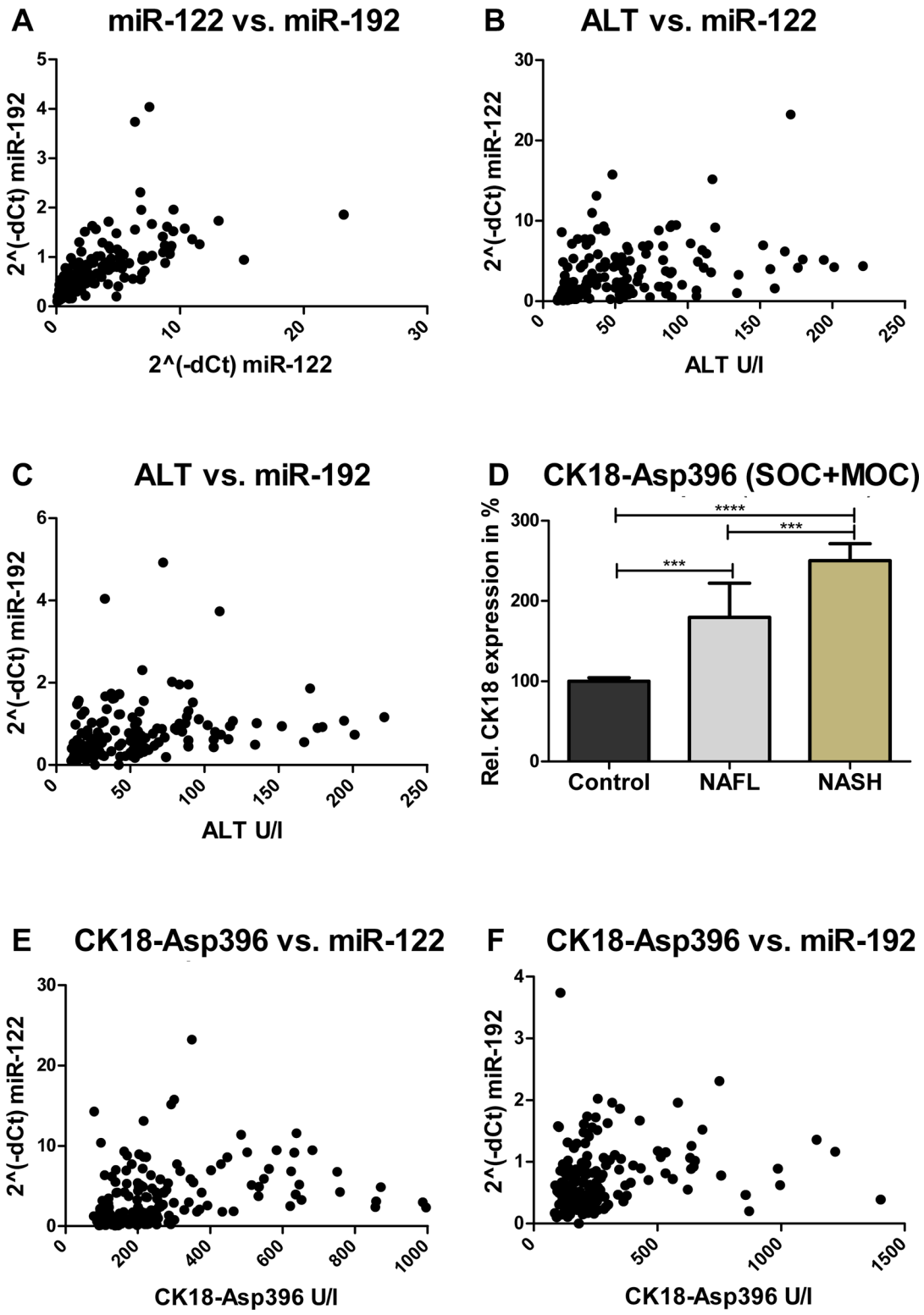

Fig 3. Correlation between different circulating microRNA levels and other serum parameters. MiR-122 and miR-192 showed a very similar profile in the different patient groups; their correlation was highly significant (A). Correlation was also analyzed in comparison to the routine serum parameters ALT and CK18-Asp396. MicroRNAs -122, and -192 could be positively correlated with ALT and CK-18 (B, C, E, F). CK18-Asp396 fragment levels increased with the development of bland steatosis and the progression to NASH (D). Detailed results are displayed in S1 Table. Correlation between variables was calculated using Spearman's Rank correlation test.

doi:10.1371/journal.pone.0142661.g003

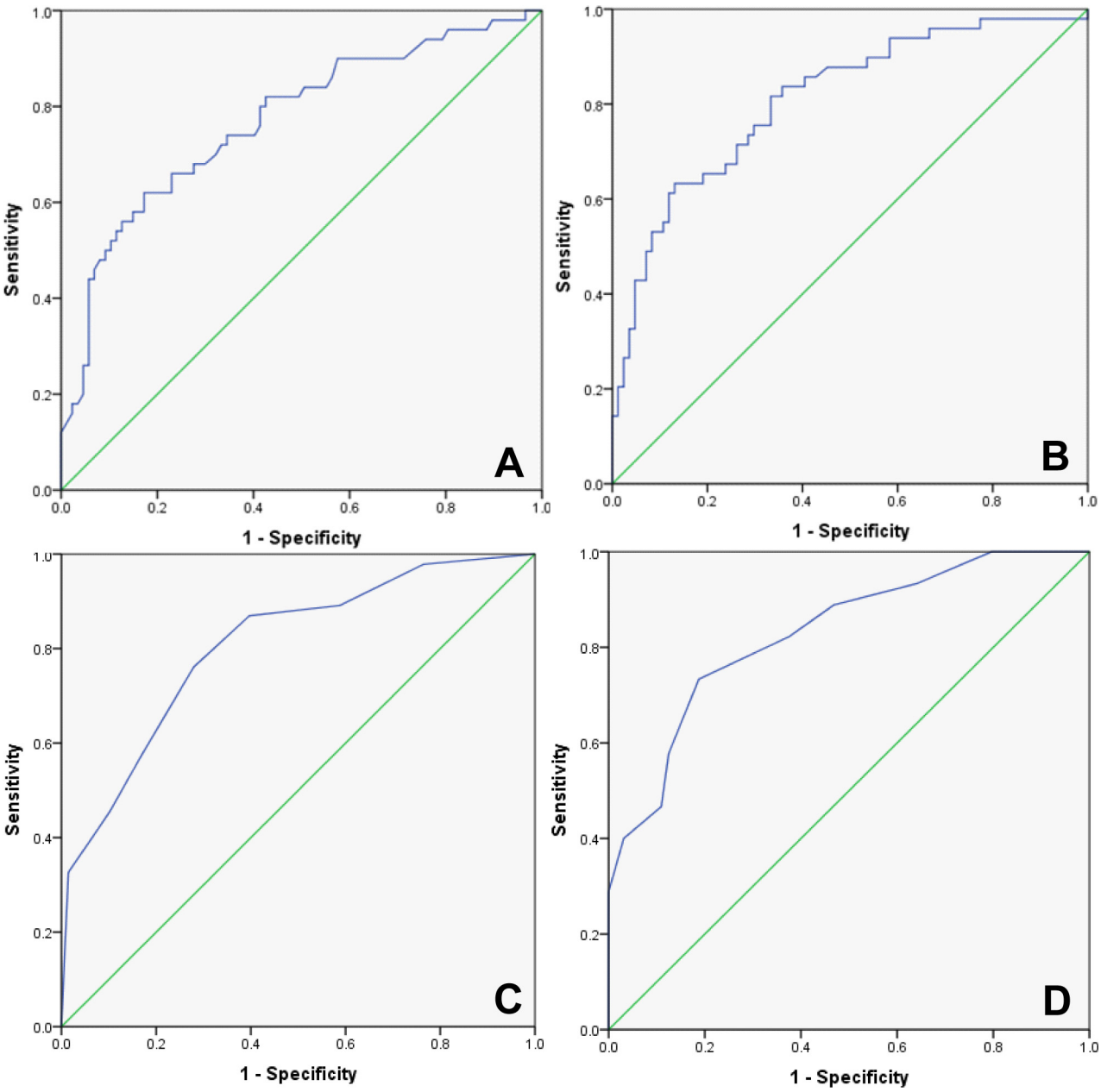

Fig 4. Diagnostic performance of microRNA level in circulation was evaluated by multivariate receiver operating characteristic (ROC) in patient group. Predictive potential of ALT (A) (AUROC: 0.77 / CI 95%: 0.685–0.854 / p: <0.001) and CK18-Asp396 (B) (AUROC: 0.81 / CI 95%: 0.733–0.837 / p: <0.001) was evaluated, represented by calculated area under ROC (AUROC). MiR-122, -192 and -21 in a combination score showed comparable results with CK18-Asp396 discrimination (C) (AUROC: 0.81/ CI 95%: 0.725–0.888 / p: <0.001). The best characteristics could be observed by combining the score with CK18-Asp396 fragment level (D) (AUROC: 0.83 / CI 95%: 0.754–0.908 / p: <0.001) to differentiate between NAFL and NASH.

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The test result variable(s): Predicted probability has at least one tie between the positive actual state group and the negative actual state group. Null hypothesis: true area = 0.5.

Discussion

Major differences for NAFL and NASH in the risk of progression to end stage liver disease, decompensation and HCC development necessitate a reliable non-invasive risk assessment in

routine clinical practice. Currently no valid method to differentiate between NAFL and NASH is available except liver biopsy with histological diagnosis. Therefore, the search for suitable serum biomarkers is pursued with high priority.

We hypothesize that circulating miRNA profiles hold great potential to predict the presence of NASH as serum biomarkers. Candidate miRNAs included miR-122 and -192, which have been identified by previous studies as to be associated with NAFL and NASH [17]. Additional candidate miR-21, which has been shown to be associated with clinically diagnosed but not biopsy proven NAFLD [33]. MiR-223 appeared as another potential biomarker for NAFLD progression since it has been shown to be significantly up-regulated in liver tissue of NASH patients (unpublished data) and has been shown to be differentially expressed in serum of HBV positive HCC patients [35].

MiR-122 represents the most abundant microRNA in adult human liver (approx. 70% of total miRNAs) and has been associated with de novo lipogenesis and lipid trafficking [36, 37]. A significant increase of miR-122 in the serum of NASH patients has been identified with positive correlation to the stages of inflammation and fibrosis [37]. Li et al. described an inhibitory effect of miR-122 on the stellate cell activation and collagen deposition within the liver indicating a link between decreased miR-122 and fibrotic liver damage [38]. In line with these observations, a decreased expression of miR-122 has been observed in liver tissue of patients suffering from NAFLD [39]. Recently, Pirola et al. had shown increased miR-122 serum levels with the manifestation of NASH [17]. Several studies have observed the decreased miR-122 expression within the liver and its increase in serum in the pathophysiological context of severe liver disease and documented a correlation between hepatocyte destruction and miR-122 export [40–42]. Recently published data also suggested that elevated levels of miR-122 in the circulation play a crucial role in regulation of genes involved in endothelial damage, as for instance the L- arginine transporter SLC7A1 [17, 43, 44].

For miR-192 comparable data are scarce. MiR-192 has been shown to share a pri-miRNA with miR-194 and to play a crucial role in diabetic nephropathy [45]. They are known to be upregulated by TGFβ1 [45], a key fibrogenic cytokine in hepatic stellate cells. Recent data by Pirola et al. indicating an increased miR-192 in liver tissue and serum of NAFL and NASH patients, further underline the role of this miRNA in different pathologies within the metabolic syndrome [17]. The present study confirms these observations indicating a significant correlation of miR-122 and miR-192 to the manifestation of NASH [17]. Most importantly, significant differences for both miRNAs between NAFL and NASH patients could be confirmed and render these two as biomarker candidates.

MiR-21 has been associated with several diseases before and it was also one of the first described oncomirs [46]. MiR-21 has also been identified as a potential diagnostic marker of viral hepatitis [47, 48]. As a new finding in our study we could show that circulating miR-21 level is significantly increased in patients suffering from NASH compared to HC and NAFL patients. An altered level in undifferentiated NAFLD has been shown before [33]. MiR-21 has also been found elevated in the context of liver fibrosis and could be correlated with higher fibrosis stages [49]. Although we do not detect such a correlation for the entire study population we assume the differences in miR-21 expression between the NASH and NAFL group may be at least partially reflected by increased fibrosis scores and fibroscan elasticities. The present data now indicate that miR-21 is further increased in inflammatory states of fatty liver disease since both cohorts showed a significant increase in NASH. The findings are in line with elevated miR-21 in HCV infected serum and hepatocytes [48, 50] which also point to a role as a marker for necroinflammatory activation in the context of viral infection.

An upregulation of miR-21 could be detected in hepatocytes after unsaturated fatty acid treatment which leads to a specific binding of miR-21 to PTEN mRNA thereby initiating its

degradation [51]. This particular study mechanistically explains decreased PTEN expression in high-fat diet treated rats and NAFLD patients and suggests miR-21 also as a key factor for the progression from NASH to HCC [51].

MiR-192 and -122 could be significantly correlated with CK18-Asp396 and ALT levels indicating that these miRNAs are released from hepatocytes during pathophysiological states associated with cell membrane impairment. Previous data indicate an increase of miR-122 in serum with apoptosis of hepatocytes caused by inflammatory damage of the liver [52] and explain the positive correlation with the apoptosis marker CK18-Asp396 fragment. A new finding here is the correlation between ALT- and miR-21 serum level. It can be hypothesized, that these miRNAs generally show an earlier increase than ALT in NASH patients, as is has been shown for miR-122 in the context of viral, drug- and alcohol-related liver disease [53, 54]. This hypothesis is strengthened by the fact, that 25% of our NASH patients with predictive miRNA/CK18-Asp396 score showed no abnormities in routine ALT level. The correlation between mir-122 and -192 is bringing up the question for a potential link in miRNA processing and regulation.

Our data on those two microRNAS are validated by the findings in a similar cohort Pirola et al. investigated earlier [17]. As the authors already investigated the potential of single miR-122 to predict NASH [17], the aim of our study consisted in combining potential microRNA- and other biomarkers to increase the diagnostic performance in the discrimination of NASH from lower risk NAFL patients. Tan et al. published in 2014 a panel of miRNAs suitable for the discrimination of NAFLD patients against healthy controls but found no difference for the different disease states of fatty liver disease [19]. Focus of this study was on the diagnosis of NASH patients within the entire group of NAFLD patients. Following this aim, for logistic regression and further analysis all NAFLD patients (NAFL plus NASH) were included. Results of our logistic regression analysis brought us to the conclusion to further analyse miR-122, -192, and -21 as potential biomarkers candidates. As CK18-Asp396 fragments has been extensively investigated as a biomarker candidate [10, 11, 14], we used this marker as a reference together with serum ALT levels. Defining the median as critical threshold to discriminate a 'lower risk' from a 'higher risk' group for every single biomarker candidate a composite score with and without CK18-Asp396 inclusion has been developed. In contrast to previous studies [17], we found CK18-Asp396 fragment levels to have a higher prediction potential compared to ALT shown in ROC analysis. Our combined scoring model had the same diagnostic performance in the discrimination of NASH CK18-Asp396 fragment serum levels. Combining the 3-miRNA profiles panel with CK18-Asp396 fragments could further improve the diagnostic performance as shown in receiver operating characteristics.

The outcome of our study bears high potential for future improvement of clinical routine, particularly for early disease. Our findings hold the potential to develop a reliable serum biomarker panel to identify patients "at risk" for NASH and thereby decrease the currently almost universal need of liver biopsies with potential complications for NASH diagnosis. Significant differences in miRNA serum levels, detectable by a decrease in Ct values, in different NAS groups together with a gradual increase starting with the earliest NAS stages fuel the assumption, that early detection and in turn treatment could be optimized with the potential use of miRNAs as candidate biomarkers.

Supporting Information

S1 Fig. Relative detection profile of four investigated circulating microRNAs in severely obese cohort (SOC). Selected circulating microRNAs show significant difference in detection between NASH and NAFL patients as well as NASH patients and healthy controls, respectively.

MicroRNA data is normalized with miR-103 and miR-425. (Displayed: Mean + SEM).
(EPS)

S2 Fig. Relative detection profile of four investigated circulating microRNAs in moderate obese cohort (MOC). Significant difference between NAFL and NASH patients could be detected for miR-21. For microRNAs 122 and 192 a discrimination between NAFLD and healthy controls was shown, whereas a significant difference between the different stages of NAFLD could not be detected. MicroRNA data is normalized with miR-103 and miR-425. (Displayed: Mean + SEM).
(EPS)

S3 Fig. Relative CK18-Asp396 fragment levels in moderate and severely obese cohort. In independent cohorts an increased abundance could be detected with the development of bland steatosis without further increase in NASH. (Displayed: Mean + SEM).
(EPS)

S4 Fig. Total number of patients scored with combined circulating microRNA /CK18-Asp396 fragment level model. The majority of definite biopsy proven diagnosis for NAFL and NASH are detected by scores 0 and 4 (sensitivity 91% and specificity 83%).
(PDF)

S1 Table. Correlation analysis with microRNA serum level. MicroRNA level in circulation correlated with routine serum parameters ALT and CK18-Asp396 and with single NAS parameters (Steatosis, lobular inflammation, ballooning and fibrosis). Also miRNAs 122 and 192 have been correlated. Analysis is applied by Spearman's rank correlation test.
(PDF)

S2 Table. Applied cut-offs for scoring model in both cohorts for miRNAs and CK18-Asp396 fragment level. Scoring of miRNA profile was performed by separating their expression in the entire NAFLD group by median, scoring '0' for lower or equal values (lower risk) and '1' for higher values (higher risk).
(PDF)

Author Contributions

Conceived and designed the experiments: AG JS PPB. Performed the experiments: PPB JS. Analyzed the data: PPB AG CH CM. Contributed reagents/materials/analysis tools: AG MR HB CM CH BM. Wrote the paper: PPB AG.

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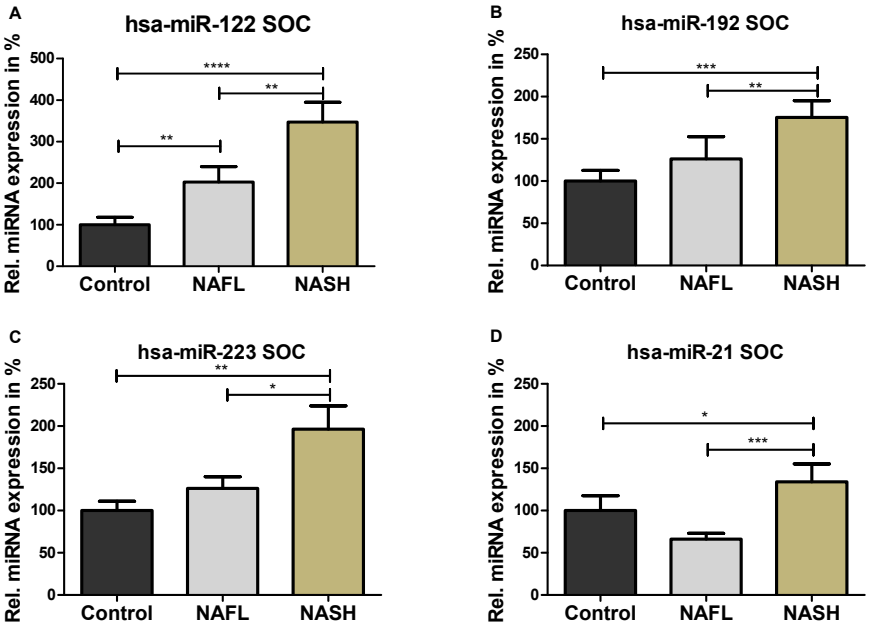
S1 Figure:



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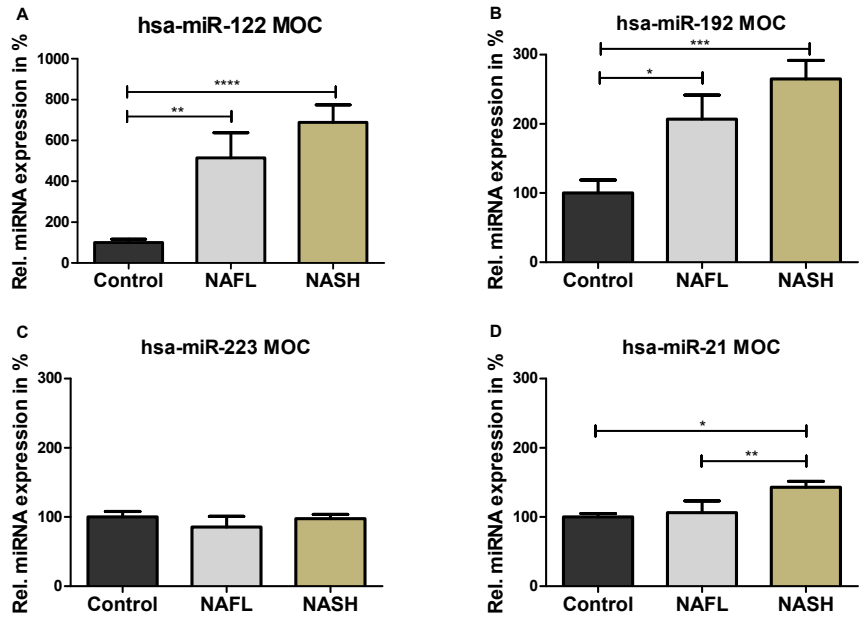

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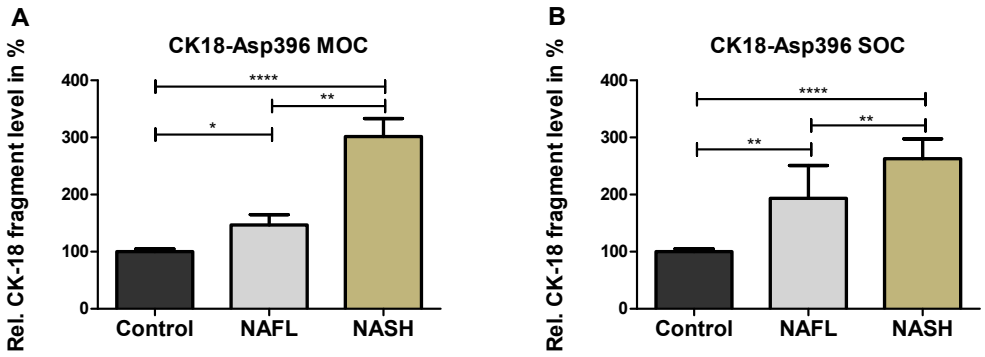

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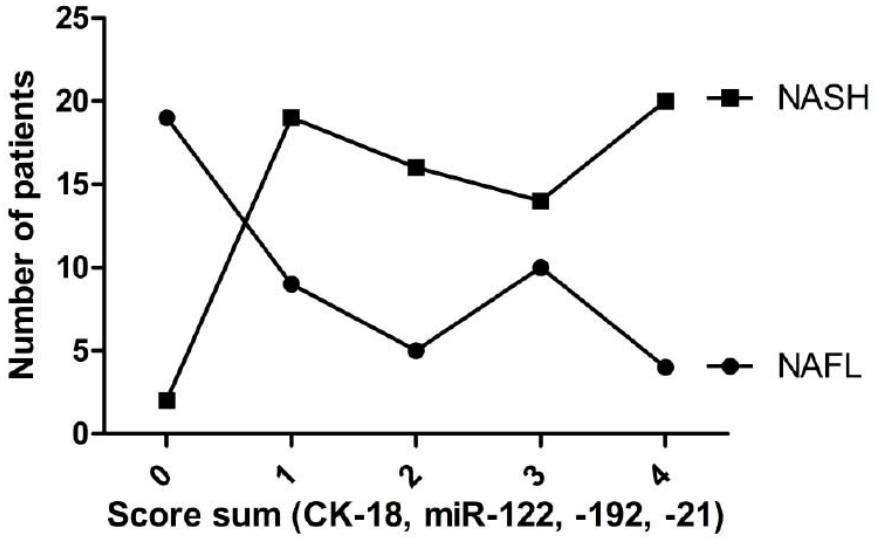

S4 Fig: Sensitivity, Specificity for NAFL (score 0) and NASH (score 4)	
Sensitivity	91%
Specificity	83%
pos. Prediction	83%
neg. Prediction	91%
Efficiency	87%

S1 Table:

S1 Table: Correlation analysis with microRNA serum level

P value (two-tailed) / P value summary	Spearman r / 95% confidence interval	hsa-miR-122		hsa-miR-192		hsa-miR-21	
ALT		< 0.0001	0.529	< 0.0001	0.4513	0.0053	0.2156
		****	0.4063 - 0.6330	****	0.3119 - 0.5717	**	0.06090 - 0.3602
CK18-Asp396		< 0.0001	0.4778	< 0.0001	0.3564	0.1388	0.107
		****	0.3569 - 0.5830	****	0.2137 - 0.4842	ns	-0.03905 - 0.2485
Steatosis		0.0002	0.3304	0.0199	0.2188	0.0014	0.2821
		***	0.1584 - 0.4829	*	0.02991 - 0.3926	**	0.1068 - 0.4404
Lobular inflammation		0.0497	0.1712	0.1426	0.1352	< 0.0001	0.3823
		*	-0.004827 - 0.3369	ns	-0.05130 - 0.3126	****	0.2220 - 0.5225
Ballooning		0.4209	0.07092	0.7144	0.03404	0.1197	0.1361
		ns	-0.1070 - 0.2444	ns	-0.1530 - 0.2187	ns	-0.04074 - 0.3047
Fibrosis		0.1101	0.146	0.3103	0.09808	0.8642	-0.01571
		ns	-0.03874 - 0.3211	ns	-0.09734 - 0.2862	ns	-0.1988 - 0.1685
hsa-miR-192 vs. hsa-miR-122		< 0.0001	0.8304				
		****	0.7751 - 0.8732				

MicroRNA level in circulation correlated with routine serum parameters ALT and CK18-Asp396 and with single NAS parameters (Steatosis, lobular inflammation, ballooning and fibrosis). Also miRNAs 122 and 192 have been correlated. Analysis is applied by Spearman's rank correlation test.

S2 Table: Applied cut-offs for scoring model in both cohorts for miRNAs and CK18-Asp396 fragment level

	Median MOC	Median SOC
miR-122 [ΔCt]	-2.259	-1.228
miR-192 [ΔCt]	0.086	0.886
miR-21 [ΔCt]	-5.377	-5.151
CK18-Asp396 [U/l]	237	242

Scoring of miRNA profile was performed by separating their expression in the entire NAFLD group by median, scoring '0' for lower or equal values (lower risk) and '1' for higher values (higher risk).

S2 Table:

4 SECOND MANUSCRIPT

Regulation of microRNAs -21, -122, -223 and -638 in the pathophysiological context of Non-Alcoholic Fatty Liver Disease

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- Manuscript is under preparation and subject to further amendments -

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Author contribution:

- RNA isolation (PB)
- Quantitative real time PCR, analysis, interpretation (PB)
- Statistical Set-Up (PB)
- Design and coordination of the study (PB)
- Manuscript writing (PB)
- Figure design and arrangement (PB)

Regulation of microRNAs -21, -122, -223 and -638 in the pathophysiological context of Non-Alcoholic Fatty Liver Disease

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List of abbreviations:
NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; IGF1, insulin-like factor 1; TNFα, tumor necrosis factor alpha; IL6, interleukin 6; miRNA/miR, microRNA; SREBPS, sterol regulatory element-binding proteins; LXR, liver X receptor; *FASN*, fatty acid synthase; *PLIN5*, Perilipin5; *JUN*, c-Jun N-terminal kinase; HC, healthy controls; AST, aspartate aminotransferase; ALT, alanine aminotransferase; RT-q-PCR, real-time quantitative PCR; SEM, standard error of mean; IHH, immortalized human hepatocytes; *HPRT*, gene of hypoxanthine guanine phosphoribosyl transferase; FFA, free fatty acids; OA, oleic acid; ANP32A, acidic (leucine-rich) nuclear phosphoprotein 32 family, member A; PTEN, phosphatase and tensin homolog; NF-κBp65, nuclear factor-kappa B p65; mTOR, mammalian target of rapamycin; UTR, untranslated region; BMI, body mass index

Abstract

Objectives: The increasing prevalence of obesity in western countries causes an elevation of the incidence of non-alcoholic fatty liver disease (NAFLD). The pathophysiological background of NAFLD is still not totally explored. The aim of this study is to identify a possible regulatory function of selected miRNAs on gene expression and their potential role as functionally relevant mediators of fatty liver disease.

Methods: In the study, expression of four miRNAs (21, 122, 223 and 638) was investigated in a human liver biopsy cohort (49 NAFLD patients and six healthy controls) via RT-q-PCR to evaluate differences in expression between patients with non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver (NAFL) and healthy controls. Potential microRNA target genes known to be relevant for metabolic changes were also investigated in this cohort. In parallel, free fatty acid treated hepatocytes were used to evaluate a possible functional relationship of miRNA and mRNA expression.

Results: For all four microRNAs of interest, a differential regulation between NAFLD patients and healthy controls could be demonstrated; liver specific miR-122 showed also a distinct expression between NAFL and NASH. Among those, miR-638 was newly shown to be differentially regulated in the context of liver disease. Expression patterns could be validated for miR -223 and -638 in cultured hepatocytes.

Conclusions: MiRNAs previously associated with metabolic or inflammatory pathways were newly assigned to either NAFL, NASH or both. These data from liver tissue imply a role of certain miRNAs as potential mediators in the progression from

bland steatosis to steatohepatitis and also appear to be of high importance to define the origin of clinically relevant pathophysiological events in fatty liver disease.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in developed countries with a prevalence of 20 to 35% [1, 2]. Non-alcoholic fatty liver (NAFL) is defined as bland steatosis and must be distinguished from non-alcoholic steatohepatitis (NASH) which is characterized by inflammatory infiltrates, ballooning and necroapoptosis. 5% to 20% of NAFLD patients develop a progression from NAFL to NASH. This is of particular importance since the presence of NASH and/or advanced fibrosis has been associated with an increased overall mortality in these patients [3, 4].

NAFLD is often defined as the hepatic manifestation of the metabolic syndrome, which is associated with insulin resistance, obesity, and type II diabetes. However, its pathogenesis is complex and still not fully understood [5].

It has been demonstrated in the past, that cytokines and oxidative stress are playing a role in the development of steatosis and more severe liver damage, such as advanced fibrosis. The progression from NAFL to NASH is also associated with the progression of the metabolic syndrome; hence associations to pro-inflammatory processes and insulin resistance could be demonstrated [6]. The linkage between type II diabetes and obesity remains in the focus. Hyperinsulinemia is causing elevated levels of insulin-like factor 1 (IGF-1), cytokines and sex steroids [7]. Also in this context a relation between chronic inflammatory state and obesity has been shown accompanied with elevated levels of pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6) [8, 9].

Since the discovery of microRNAs (miRNAs) in 1993 [10], scientists demonstrated a regulative function in various gene expression pathways. These small RNAs of 19 to

23 nucleotides in total length regulate gene expression acting usually at posttranscriptional level. In majority (80%), their sequence is located in non-coding intronic sections of the genome [11, 12]. The outcome of several studies also reported a potential role of miRNA regulation in the pathophysiology of NAFLD [8, 13, 14]. This has also lead to a number of approaches, to use alterations of miRNAs in circulation as non-invasive diagnostic tools [15, 16]. To define miRNAs involved in the progression and development of NAFLD is baring high potential for future therapeutic perspectives, as an inverse treatment with respective miRNAs or anti-miRNAs (antagomirs) in affected tissues might lead to positive treatment responses [14, 17, 18].

Various genes involved in the pathogenesis of NASH have been described in the past, also in line with respective targeting miRNAs. These include three isoforms of sterol regulatory element-binding proteins (SREBPs) which are transcription factors with important roles in triglyceride and cholesterol synthesis [19, 20]. For SREBP1 and 2 a regulatory role of miR-24 could be demonstrated in a murine study [8]. The cholesterol sensor liver X receptor (LXR) is involved in the metabolism and absorption of sterols. Yoshikawa et al. described their promoting role in the development of NASH by an indirect activation of SREBP-1c [21]. Joseph et al. described a direct involvement in regulation of lipid synthesis, by interacting with genes such as for instance fatty acid synthase (*FASN*) [22]. More relevant data to picture and connect miRNA expression and related transcript regulation is scarce.

As miRNAs have been associated previously with metabolic or inflammatory pathways, the aim of this study was to define relevant miRNAs which could be newly assigned to either NAFL, NASH or both. Another goal was the functional assignment in central signaling pathways. The hypothesis of this study is that the selected four investigated miRNAs are playing a particular role in the development and progression

of NAFLD and have a regulative function on the expression of *FASN*, *PLIN5* (Perilipin 5) and *JUN* (C-Jun). The identification of miRNAs of relevance as potential mediators in the progression from bland steatosis to steatohepatitis and also in the definition of the origin of clinically relevant pathophysiological events in fatty liver disease might be of high importance for potential treatment or diagnostic options within the future.

Material and Methods

Study design, patients and healthy controls

In total, 49 NAFLD patients and 6 Healthy controls (HC) >18 years were enrolled between July 2007 and August 2014. All of them were characterized thoroughly at University Hospital of Zürich and the University Hospital Würzburg. All patients had histology proven NAFLD (28 NAFL/ 21 NASH) and were diagnosed with a non-alcoholic fatty liver disease first by typical ultrasound findings with a sonographic NASH-score[23]. All liver samples were diagnosed by an experienced pathologist.

Table 1 shows baseline parameters of NAFLD patients and controls. Subjects with significant alcohol consumption (females > 20g/day and males > 30g/day) and any pre-existing liver disease were excluded. Healthy control subjects were examined prior to living liver donation and had no evidence or history of liver pathology. Laboratory analysis showed no elevation for AST and ALT, as also no history of coronary heart disease, hypertension, valvular disease, any arrhythmia or systemic disease for inclusion in the study.

The controls and patients were matched based on gender, and ethnicity. From all tissue samples, total RNA fraction was isolated for further analysis. All liver biopsies have been obtained either percutaneously or during bariatric surgery.

The study was approved by the Würzburg University and Zürich cantonal ethics committee. Written informed consent was obtained from each patient prior to participation. The entire study was conducted in accordance with the declaration of Helsinki.

Liver tissue and cell culture samples; RNA isolation

From routine liver biopsies a 5mm tissue cylinder was collected in cryotubes (TPP, 2ml), directly snap frozen in liquid nitrogen and stored at −80°C until use. 25mg of tissue were cut from frozen biopsy sample and homogenized with pistil in 700 ml QIAzol lysis reagent (Qiagen). For functional assays cultured cells were harvested after 56 h with cell scratcher and directly taken up in 700 ml QIAzol lysis reagent. From homogenate, total RNA, including small RNAs was extracted with miRNeasy Mini kit (Qiagen) according to the Qiagen Supplementary Protocol for purification of small RNAs from tissue and cells, and finally eluted in a 50µl volume of nuclease-free water. RNAs were quantified by fluorometer and spectrophotometer (Nanodrop, Thermoscientific).

MiRNA/mRNA candidate selection; cDNA-synthesis and RT-q-PCR

Several miRNAs were defined as candidates of interest, as they have been reported to be associated with liver disease or other metabolic manifestations in present literature (miRs-21, -122, -223). MiR-122 is the most abundant miRNA and specific in liver tissue [24, 25]. MiRNAs -21 and -223 have been reported to be associated with NAFLD, hepatocellular carcinoma or type II diabetes [26-28]. MiR-638 was also included as it showed a significant difference between two small groups NAFL and NASH patients in a pilot study using an unbiased array approach (data not shown). Quantification of miRNA expression for candidate miRNAs was performed by RT-q-PCR. cDNA transcription was performed by two different protocols: For three miRNAs (MiR-122, -21, -223), RNA (500ng) was reverse-transcribed in 20 µL

reactions using the NCode™ Vilo™ miRNA cDNA synthesis kit (life technologies) according to user manual (Version A, 12 February 2009). The cDNA was diluted 1:10 and amplified in 10 µL PCR reactions using Express SYBR GreenER™ miRNA RT-q-PCR Kit (life technologies) according to user's manual (Version A, 12 February 2009). The amplification for single RT-q-PCR for each of the ten miRNAs was performed on 7900 HT real-time PCR instrument (Applied Biosystems) using specific designed miRNA primers (Microsynth, Switzerland) to quantify miRNA. For miR-638, TaqMan Small RNA Assays, MicroRNA Assays (TaqMan®) was performed. Reverse Transcription was done with TaqMan MicroRNA Reverse transcription Kit (life technologies), and for RT-q-PCR, Taqman Universal PCR Mastermix II (2x), no UNG, was used (all material life technologies) according to user's manual (Version 01/2011). RT-q-PCR for miR-93 (as intracellular reference miRNA [29-31]) was performed for both methods.

Three gene transcripts of metabolic and inflammatory pathways were defined to be candidates of interest chosen by the selection criteria (i) potential target of the relevant miRNAs and (ii) pathophysiological relevance in liver metabolism. The candidate genes were *FASN* (encoding fatty acid synthase) and *JUN* (encoding c-Jun N-terminal kinase); selected as they are potential targets of miR -21, -122 and -223. *PLIN5* (encoding perilipin 5) is a predicted target for the newly described miRNA 638 and is known to be involved in lipid droplet accumulation [32]. Quantification of mRNA expression for candidate mRNAs was performed by RT-q-PCR with whole cohort in one run. cDNA transcription was performed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions (Vers. 11/2010). RNA (500ng) was reverse-transcribed in 20 µL reactions. The cDNA was diluted 1:10 and amplified in 10 µL PCR reactions using 20

× Gene Expression Assay and 2× Gene Expression Master Mix (both: TaqMan®) according to user's manual (Version 11/2010). RT-q-PCR was performed on 7900 HT real-time PCR instrument (Applied Biosystems). mRNA data was normalized with human HPRT gene.

Primer

Primer for Express SYBR GreenER™ miRNA RT-q-PCR Kit (life technologies) were designed according to manufacturer's manual (life technologies, Version A, 12 February 2009), melting temperature was evaluated by Oligo Analyzer 3.1 (IDT, Iowa, USA) and oligo assembling was performed by Microsynth (Switzerland).

miRNA	**Primer-Sequence**	**T melt (°C)**
miR-122	GCC GCA ACG CCA TTA TCA CAC TAA ATA AAA AA	60
miR-21	GCC CGT AGC TTA TCA GAC TGA TGT TGA A	60.1
miR-223	CCC CTG TCA GTT TGT CAA ATA CCC CA	60.7
miR-93	GCA AAG TGC TGT TCG TGC AGG TAG	60.7

Cell culture

Immortalized Human hepatocytes (IHH) were cultured in WilliamsE Medium (Sigma Aldrich), containing 10% FCS (TPP, Switzerland), 2mMol/L L-Glutamin (Sigma Aldrich), 100U/ml Penicillin/Streptomycin (Sigma Aldrich), 20mU/L Insulin solution and 50 nMol/L Dexamethason (Sigma Aldrich). Briefly, $5x10^5$ human hepatocytes were plated in 6-well collagen I coated clear plates. After 24 hours, hepatocytes were treated with either the control medium (WilliamsE as described above), Vehicle control medium (control medium containing 0.25% Isopropanol) or medium

containing oleic acid (Carl Roth, Germany) (control medium containing Isopropanol and oleic acid in concentrations 50, 100, 250, 500mM). Cells were cultured for another 32 hours before harvesting. All concentrations were applied in four independent assays. RNA isolation was done as described above. mRNA data is normalized with human *HPRT* gene.

Statistics

Statistical analyses and graphs were performed and created with Prism5 (GraphPad Software, La Jolla, CA).

P-values < 0.05 were considered statistically significant (*< 0.05, **< 0.01, ***< 0.001). Quantitative data were expressed as mean ± standard error of mean unless otherwise indicated. For relative miRNA expression, miR-93 expression was considered for normalization [30, 31]; 2-Δ Ct –method was used. To compare miRNA expression, Mann-Whitney U test was applied. Correction for multiple testing was not applied, as the focus of the study was rather exploratory.

Results

Baseline patient characteristics

Data from 55 participants were included in the study (28 NAFL, 21 NASH, 6 HCs) (Table 1). The groups did not differ in age, sex, race/ethnicity, or alcohol intake. Patients with NASH and NAFL, had higher BMI than HCs. Liver transaminases, fasting glucose, triglycerides and total cholesterol were higher in patients than in HCs. For triglycerides and total cholesterol the values were higher in NAFL patients than in the NASH group.

Expression data in whole cohort

RT-q-PCR showed significant differences in expression patterns for all four miRNAs of interest.

MiR -21 showed an increase in expression for NAFLD patients compared to controls ($p= 0.0042 - 0.0053$), however statistical significance was not obtained between the subgroups of NASH and NAFL (Fig. 1A).

MiR-122, which has been demonstrated in the past [13] to be downregulated in NAFLD patients, could be validated in the investigated cohort, but also a significant differentiation between NAFL and NASH could be observed ($p = 0.0256$). MiR-223 and -638 showed no differential expression between bland steatosis and steatohepatitis; however the expression showed a significant difference comparing NAFLD and healthy tissues (Fig.1C/D).

MRNA expression

The expression of the three target genes of interest was analyzed via RT-q-PC. For all three genes an upregulation could be observed (Fig. 2). *FASN* and *JUN* showed a significant expression difference in comparison to the healthy control group. However, an expression difference between NAFL and NASH could not be observed (Fig. 2 A/B). For *PLIN5*, a marginal difference could be obtained however without statistical significance (Fig. 2 C).

Functional assays in cell culture

In an in vitro assay, IHH cells were treated with free fatty acids (FFA) to simulate the condition of hepatic lipid accumulation and lipotoxicity. Different FFA concentrations were applied in independent assays. First, the expression of target miRNAs was measured via RT-q-PCR, to verify if similar expression patterns as in human samples could be obtained. MiRNA 223 showed an upregulation when comparing treated and untreated cells. Only in cells treated with FFA in high concentrations (250/ 500 mM) a significant difference could be observed ($p = 0.0286$) (Fig. 3A). The direction of regulation is corresponding to the human liver biopsy cohort.

For miR-21, a downregulation could be observed, which stands in contrast to observations in the patient cohort on the first view. For the treatment with 100 and 500 mM of FFA, this expression change was significant ($p = 0.0286$) (Fig. 3B).

MiR-638 showed for all treatment conditions lower expression. However, a significant change could only be observed in treatment with the lowest concentration (50 mM) ($p = 0.0286$) (Fig. 3C). For miR-122, no change in expression could be observed in treated hepatocyte cultures (Fig. 3D).

MRNA expression for the three genes of interest has also been determined in the same cell culture assays. *JUN* transcripts did not show any change in abundance in cells treated with high concentrations of FFA (250/ 500mM). A visible trend towards upregulation, comparable to the expression pattern in human tissue, could be observed in assays treated with low concentrations of FFAs but statistical significance could not be reached (p (100mM) = 0.13) (Fig 4A).

Evaluation of *FASN* expression also showed no changes in assays treated with low concentrations of oleic acid. In the highest concentration a visible tendency of higher expression could be determined (p=0.2) (Fig 4B) which would be likewise as in patient cohort.

For *PLIN5* the expression pattern in cell culture showed the most constant increase depending on FFA concentration reaching a plateau with higher FFA concentrations (≥100mM) (Fig 4C).

Discussion

As the development and progression of NAFL and NASH is not entirely understood on the functional level, the present study investigated the tissue expression of four miRNA candidates in healthy livers and the different stages of fatty liver disease. The exploratory approach included also an analysis of potential pathophysiologically relevant mRNA targets of these miRNAs and a first functional analysis in an in vitro model.

MiR-21 was described to be one of the first oncomirs, hence a lot of its targets are known and described as tumour suppressor genes. Prominent examples would be acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A), phosphatase and tensin homolog (PTEN) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A member 4 [33, 34]. In this study, an upregulation in NAFLD patients could be detected. Similar changes could also be observed by Vinciguerra et al., who described an upregulation in hepatocytes after unsaturated fatty acid treatment, mediated by NF-κBp65/ mammalian target of rapamycin (mTOR)-complex. [35]. In contrast to this study, our observations show a downregulation of miR-21 in cultured hepatocytes. A possible explanation could be the longer incubation with oleic acid in our approach. As Vinciguerra et al. described a fast increase of miRNA expression after 24 hours with low concentrations of FFA (50mM), a fast alteration in the beginning followed by a decrease caused by the binding to PTEN transcripts can be suspected [35] after 32 hours. Also possible would be an intracellular mechanism setting the miRNA free during cell degradation in the extracellular matrix as it is known for miR-122 [13, 25]. The presented results

further underscore potential of miR-21 as a key mediator of the metabolic syndrome and NAFLD.

MiRNA 122 is the most abundant miRNA within the liver and was also the first with a functional link to lipid homeostasis and metabolism [36]. Cheung et al. showed a highly significant downregulation of miR-122 in NASH patients [13]. This particular downregulation can also be assessed from the presented data, whereas in the present study an additional significant difference between NAFL and NASH patients could be detected. Cheung et al. further assessed the effects on its targets by overexpression and silencing in an in vitro study and observed a number of effects on target genes in protein translation, cell proliferation, inflammation, apoptosis, oxidative stress and metabolism. The functional impact on lipid metabolism caused by decreased miR-122 [13] contributed to the hypothesis that these pathophysiological effects could be mediated by an upregulation of the miR target genes *JUN* (inflammation) and *FASN* (lipogenesis). As predicted, an upregulation of miR-122 could be observed in human liver tissue of NAFLD patients but could not be replicated in the IHH model (Fig. 4). The significant difference in miRNA expression between NAFL and NASH patients could not be seen at the level of potential mRNA target transcripts. An explanation would be the fact that often several miRNAs are targeting one gene transcript [37]. Nevertheless, the correlation of the expression pattern between miR-122 and its mRNA targets in human liver tissue might be another hint for a regulative function. An in-depth evaluation of miRNA and mRNA expression, which is already subject of mathematical remodeling analyses in the field will bring new light in the regulative link between miR-122 and its targets genes.

MiR-223 has been investigated not only in the context of hepatocellular and colorectal carcinoma [38, 39] but also regarding cholesterol uptake and homeostasis

[40, 41]. However, it has not been investigated in human fatty liver disease to date. In this study an upregulation of the miRNA could be observed in the human study cohort and also in the cultured hepatocyte model. These results are in full accordance with a study conducted by Shpyleva et al. showing an altered hepatic expression in a rodent model of fatty liver disease[42]. The fact that miR-223 is also targeting the investigated genes *JUN* and *FASN* is another example for the fact the gene expression is not only regulated by one particular miRNA [37].

The newly described miRNA 638 has also not been investigated in the context of metabolic manifestations before at all. It came into the focus of interest when it showed a different regulation in a pilot study comparing two groups of NAFL and NASH patients. Also, one of its predicted targets is perilipin5 (*PLIN5*), a member of the Perilipin family which is encoding for the lipid storage droplet protein5 (LSDP5). Members of this protein family are essential for coating intracellular lipid storage droplets as well as their protection from lipolytic degradation[32]. The prevention from lipolysis is a crucial step in the development of hepatocellular steatosis. The expression of perilipins is highly abundant to adipocyte and steroidogenic cells, as for instance hepatocytes [43-45]. In the extended context of liver diseases, there is no relevant data existing on miR-638, until very recently Kumar et al. showed an antiviral potential of the miRNA in the context of HBV infections [46]. Other studies showed different regulations in colorectal cancer patients [47, 48] but lacking a clarification of its functional pathways. In this study, however, a difference in expression within different stages of NAFLD could not be validated. Although a clear downregulation in patients compared to controls could be observed, a similar behavior appeared also in cultured hepatocytes treated with FFA. The clear significant effect on *PLIN5* was lacking in the tissue cohort, even when direction of regulation was as expected. In

IHH cells this effect was significant for treatment with low concentrations of FFA (50-100mM), which might be evidence for a dependence on the degree of steatosis. The verification of these predicted target genes, suggesting a regulatory role in the context of metabolic manifestations or a potential contribution to NAFLD development represent one goal of further investigations within this field.

The results of this study are giving clear evidence, that the four miRNAs of interest are playing an important regulatory role in the context of NAFLD in general and in part even discriminate bland NAFL from NASH. The present data at least give a clear hint on their influence on potential inflammatory and lipogenic target genes. Only a final analysis also considering a broad miRNA/mRNA expression data overview based on mathematical correlation modeling will bring light in the multi-regulated miRNA/transcript conjunction. Future unbiased studies, such as an ongoing array study in our group will allow such calculations and contribute to the investigation of further functional miRNA/mRNA regulatory relationships in the pathogenesis of NAFLD.

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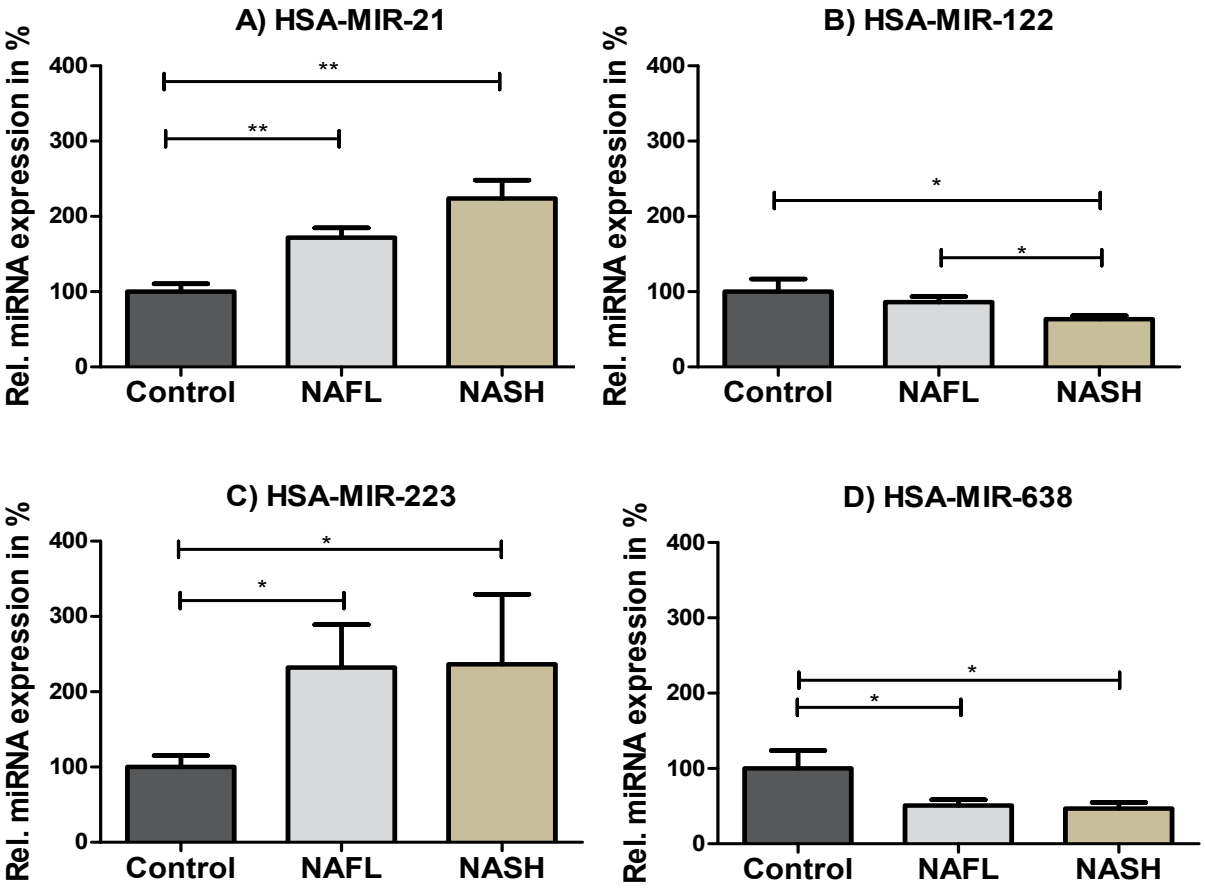
Tables and figures

Table 1:

Tab. 1: Full Cohort			
n=55	HC (n=6)	NAFL (n=28)	NASH (n=21)
Sex (m/f)	3/3	12/16	10/11
Age	37.2 ± 12.4	46.0 ± 12.8	49.4 ± 11.0
BMI	23.7 ± 4.9	36.3 ± 11.4	34.9 ± 11.0
Fasting glucose [mmol/l]	5.1 ± 0.2	5.9 ± 1.5	7.6 ± 3.9
Triglycerides [mmol/l]	0.7 ± 0.2	4.1 ± 4.8	2.5 ± 1.5
Cholesterol total [mmol/l]	4.7 ± 0.9	6.8 ± 3.9	5.1 ± 1.6
AST [U/l]	22.8 ± 4.7	46.2 ± 27.7	64.4 ± 31.9
ALT [U/l]	23.5 ± 18.1	68.9 ± 44.1	101.7 ± 61.0

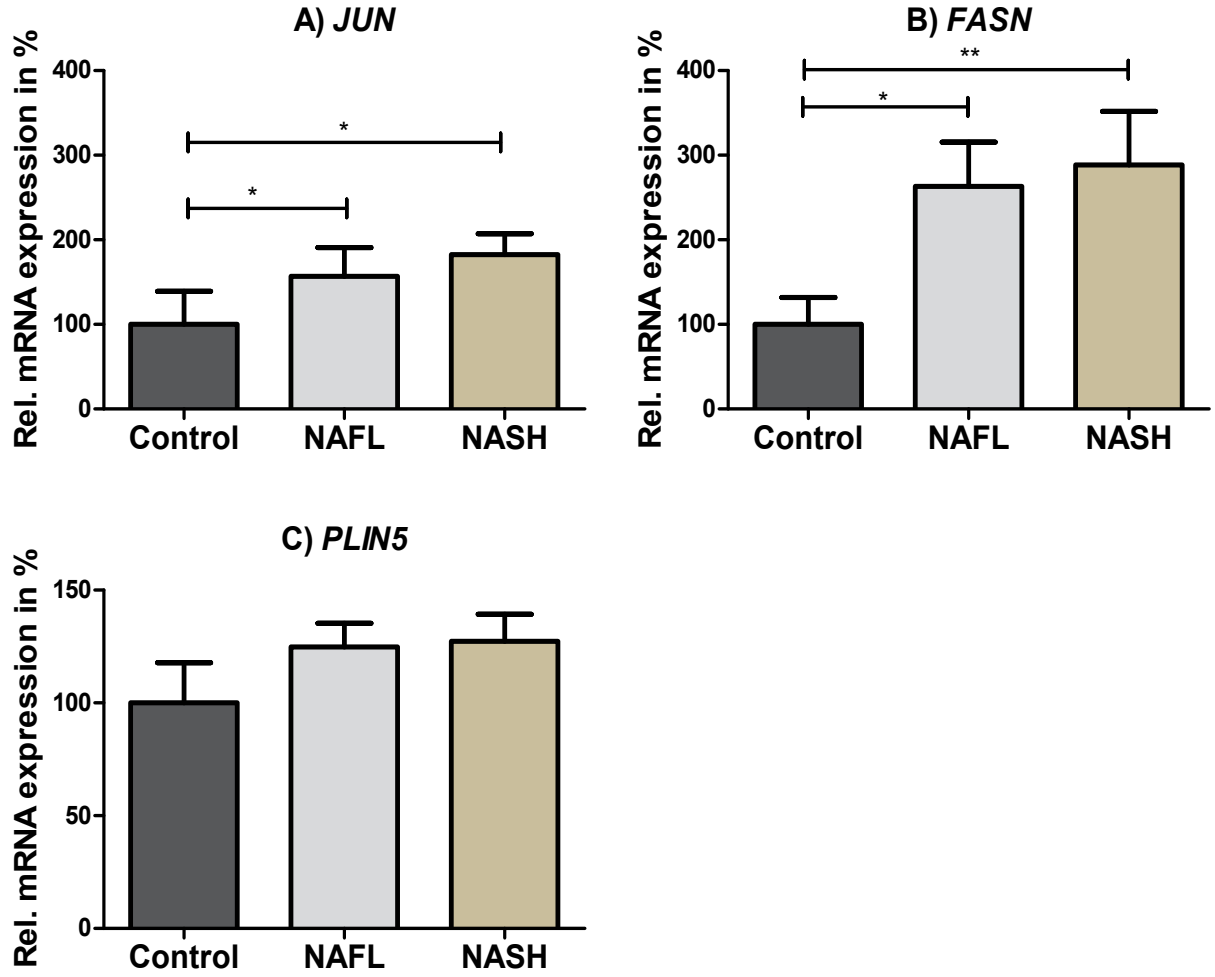
Baseline participant characteristics in NAFLD- and control cohort. Values given are expressed as mean ± standard deviation unless indicated differently.

Figure 1:



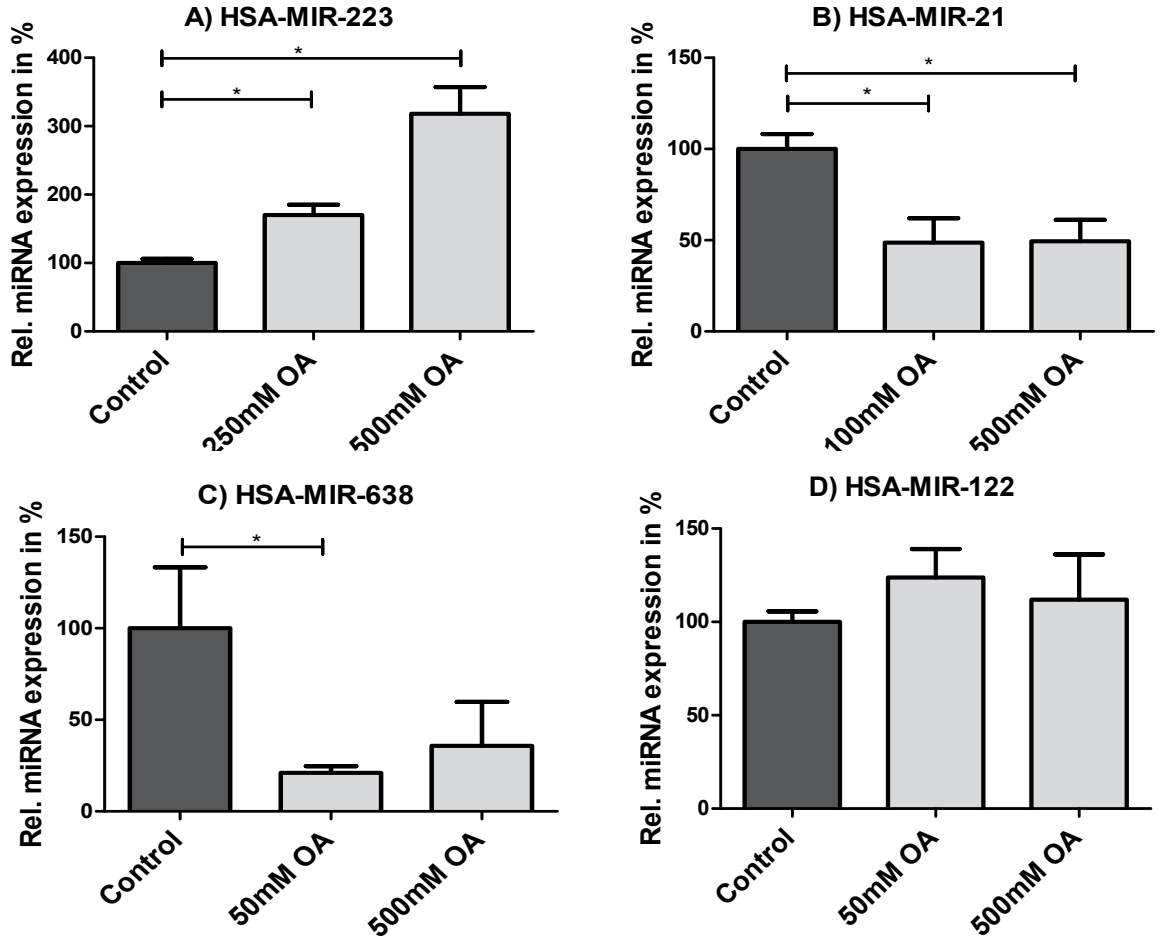
Relative expression patterns of four investigated microRNAs. Pooled data from full study cohort. Displayed microRNAs show significant difference in detection between NASH/NAFL patients and healthy controls. MicroRNA -122 (b) discriminates between bland steatosis and NASH patients. MicroRNA data is normalized with miR-93. (Displayed: Mean + SEM)

Figure 2:



Relative expression patterns of three investigated gene transcripts. Pooled data from full study cohort. Displayed genes *JUN* (A) and *FASN* (B) show significant difference in detection between NASH/NAFL patients and healthy controls. *PLIN5* shows expected direction of regulation without reaching level of significance (C) (p= 0.25-0.31). mRNA data is normalized with human *HPRT* gene.

Figure 3:



Relative expression patterns of four investigated microRNAs in cultured hepatocytes (IHH). Pooled data from four independent assays. $5x10^5$ human hepatocytes were treated after 24 hours with control medium, vehicle control medium or medium containing oleic acid (concentrations 50, 100, 250, 500mM). Cells were cultured for another 32 hours before harvesting. Three of four displayed microRNAs show significant difference in expression between treated cells and controls (untreated). MicroRNA -122 (D) shows no expression difference in all assays. MicroRNAs -223 (A) and -21 (B) show a relevant difference only in assays treated with high concentrations, whereas a significant change for miR-638 could only be detected in the lowest concentration (50 mM; B). MicroRNA data is normalized with miR-93. (Displayed: Mean + SEM).

Figure 4:

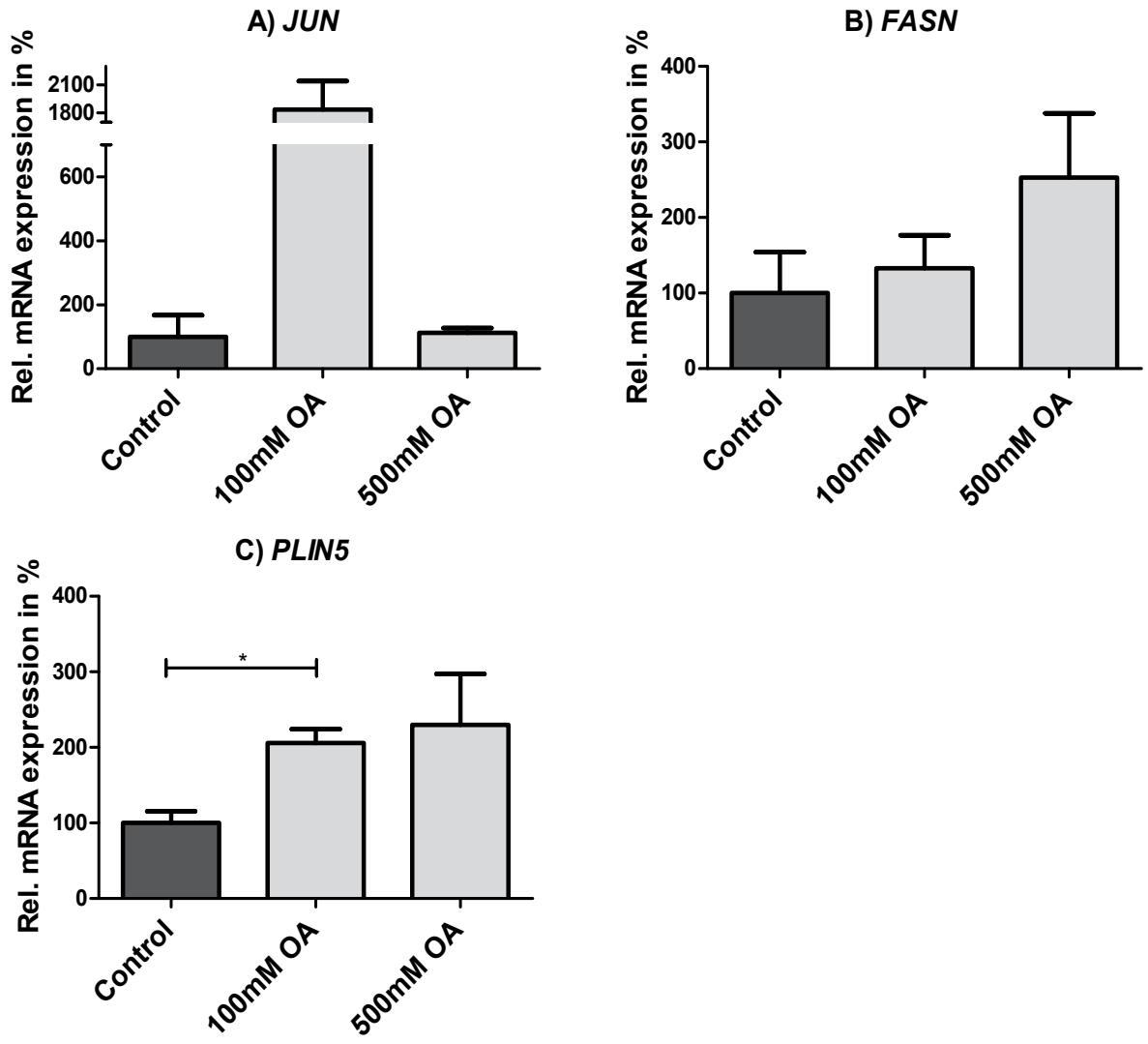


Fig. 4: Relative expression patterns of three investigated gene transcripts in cultured hepatocytes (IHH). Pooled data from four independent assays. $5x10^5$ human hepatocytes were treated after 24 hours with control medium, vehicle control medium or medium containing oleic acid (concentrations 50, 100, 250, 500mM). Cells were cultured for another 32 hours before harvesting. For PLIN5 a significant difference in expression could be detected between treated cells (100mM) and controls (untreated) (C). mRNA data is normalized with human HPRT gene. (Displayed: Mean + SEM).

5 DISCUSSION

The prevalence of NAFLD represents a rising health problem in Western countries. Major differences for NAFL and NASH in the risk of progression to end stage liver disease, decompensation and HCC development necessitate a reliable non-invasive risk assessment in routine clinical practice as also the pathophysiology of the disease is not entirely clear. The sole fact, that there is a differential expression of some miRNAs in the serum of NAFLD patients is not new[97, 278]. Also an involvement of miRNAs in the different states of the disease within liver tissue samples could be demonstrated[139]. However, clarification of the functional mechanisms of miRNAs within the pathophysiology of the disease is often missing. Also a clear separation of NAFL and NASH patients is a major weakness in most previous studies. At the end, miRNAs and other potential serum biomarkers have been investigated, but a candidate showing a specific and satisfying performance as diagnostic biomarker could not be defined[14].

The results presented in this work describe miRNAs' miR-192 and miR-122 role as discriminating serum biomarkers. These miRNAs have been observed before to have a prognostic potential in the discrimination between NAFL and NASH[97]; which could be validated in different cohorts. Furthermore, miR-21 could be described as a new miRNA candidate significantly upregulated in serum of NASH patients only, with the conclusion to be a relevant marker for NASH diagnosis. As miR-21 has been investigated to play a role in the context of more than one disease[240], obviously a

combined model was designed, pooling the expression data of the three noted miRNAs with CK-18 fragment level. A simplified scoring model was evaluated for its diagnostic specificity and efficiency in NASH detection in a multivariate analysis.

Also, an exploratory approach should expose whether miRNAs have differential expression patterns in liver tissue of NAFL and NASH patients and healthy controls. This part of the study was aiming to detect potential functional mechanisms within the progression from healthy liver into bland steatosis and further into the state of inflammatory manifestation on four candidate miRNAs. Within the study, expression data of a human biopsy cohort consisting of NAFL and NASH patients and healthy liver samples was compared. In parallel, the corresponding mRNA expression profile of three regulative target genes of analyzed miRNAs, all described to be highly relevant in liver metabolism and inflammation was generated; with the implication to bring new light in respective direct regulative interaction of these miRNAs. First approaches to analyze pathophysiological consequences on central metabolic and inflammatory pathways have been undertaken and build the groundwork for further functional assays in an in vitro model.

5.1 Rationale of the study design

In one part of this study the focus is on the diagnostic potential of miRNAs in fatty liver disease, respectively in the discrimination of NAFL from NASH. This could be obtained by investigating the expression of a panel of microRNAs in two

independent cohorts consisting of NAFL and NASH patients and healthy controls, each. Importantly, for a clear differentiation between NAFL and NASH, all patients included have previously undergone a liver biopsy which was examined by an experienced pathologist. The panel consisted of miRNAs recently published to be differentially regulated between NAFL and NASH patients[97] as well as of miRNAs which came to the focus of interest due to differential regulation in liver tissue. As the two cohorts were assembled in different degrees of obesity (moderate and morbid obesity), the study had more than one aim. The first was to verify miRNA expression presented in current literature in general, but also to see whether the expression is dependent on the degree of obesity, which has not been observed before. The second aim was to investigate the role of selected miRNAs, which showed detectable expression patterns in hepatic tissue, as potential biomarkers for the discrimination of NASH patients in serum. As more than one miRNA showed a significant difference in appearance between NASH and NAFL patients, the assessment of a simplified combined scoring system was self-evident. When the combination of miRNA levels showed no improvement in comparison to established prediction factors, the miRNA data was combined with measurements of CK-18 fragment level. CK-18 is an apoptosis biomarker which holds potential[92, 93], but is not yet recommended as solid marker for differentiation between NAFL and NASH by current clinical guidelines[14, 92, 93]. The resulting data was evaluated on its diagnostic efficiency and specificity in a multivariate receiver operating characteristic (ROC)

and compared to other existing prognostic markers, as for instance the altered levels of liver transaminases.

In the functional approach applied within this work in liver tissue, expression of four miRNA candidates was investigated in a human study cohort. A crucial step was the initial critical evaluation of patient data to define groups with the highest possible level of homogeneity.

Based on the fact that miR-122 is the most abundant and frequently investigated miRNA within the context of liver diseases[203], it was included as a candidate of interest. Our motivation here was to investigate a well-established target for verification but also one which still bears a high potential for the detection of new regulatory mechanisms.

In parallel - following the aim of this study to identify functional mechanisms of respective miRNA - total RNA of patients included in the same study cohort which was utilized for miRNA quantification was evaluated to obtain expression data of three target gene candidates.

To follow this exploratory functional approach, human liver cell lines were treated with FFA, simulating the conditions of a deposition and accumulation of lipid droplets. Immortalized human hepatocytes (IHH) were chosen to receive 32 hours FFA treatment to induce ‘steatotic’ conditions. The first aim of establishing this model was the verification of expression patterns of miRNAs and mRNAs observed in human tissue samples. The achieved verification for most mi- and mRNA

expression is representing the groundwork for further investigations to proof and demonstrate functional hypotheses, for instance via miRNA knockdown and overexpression.

5.2 Summary of results

The part of the study investigating the diagnostic role of miRNAs in NAFLD, the obtained results can be summarized as following: selected microRNAs' expression levels were analyzed in serum of two independent human patient and control cohorts; one in a morbid and one in a moderate obese condition. MiRNAs 122 and 192 have been identified before to be differentially expressed in NAFL and NASH patients[97]. Our results could validate these results; however we could observe a difference between the two cohorts, suggesting an influence of body mass on miRNA regulation. As a new finding, we could observe a significantly elevated expression of miR-21 in NASH patients, exclusively. The miRNAs expression was correlated among one another, presenting an interesting correlation between miR-122 and miR-192. The correlated expression of miRNAs to other potential prognostic NAFLD biomarkers (ALT and CK-18 fragments) suggested promising parallels. A binary logistic regression model was used to evaluate the contribution of miRNA expression on NAFLD progression (NAFL to NASH). To combine the relevant parameters, a simplified scoring model was designed, pooling miRNA- and CK-18-fragment levels. The diagnostic performance was evaluated by using receiver

operating characteristics (ROC) and showed an improvement in comparison to score parameters in exclusive use and to other prognostic markers.

In the part of the study undertaken in human liver tissue we could demonstrate differential regulation of four microRNAs in the context of NAFLD: MiR-21, -223 and -638 showed alterations in comparison of patients to healthy controls. For miR-122, an additional significant difference could also be detected comparing NAFL and NASH patients.

In concern of the evaluation of the total RNA in hepatic tissue of patients, the obtained results are the groundwork to generate a broad overview of mRNA expression within the different stages of NAFLD. Future unbiased studies, such as an ongoing array study will allow such calculations and contribute to the investigation of further functional miRNA/mRNA regulatory relationships in the pathogenesis of NAFLD. In mathematical and statistical network analysis, pathway analysis and regulation models are going to be generated, which will give a broad overview on miRNA dependent pathway regulation in the context of NAFLD progression.

For advanced functional analysis an in vitro assay could be established, simulating the conditions of hepatic fat accumulation. A change of miRNA expression comparable to patient data could be detected for miR-21, -223 and -638.

As four miRNAs were in the focus of further interest, the expression patterns of selected and with NAFLD associated target genes - *JUN*, *FASN* and *PLIN5* - were

also investigated in liver tissue as well as in cultured cells. For miR of interest 638 a similar expression in hepatic tissue and cultured cells could be observed, the effect on its potential target gene *PLIN5* could not be fully correlated: whereas in IHH cells an inverse correlation of expression was obtained, in liver tissue statistical significance could not be shown. For *JUN* and *FASN*, the expression data detected in vivo and in vitro was highly comparable: A higher rate of detected transcripts in patients and in FFA treated cells could be observed in comparison to healthy controls and untreated cells.

5.3 MiRNAs as potential diagnostic markers in NAFLD

The prevalence of NAFLD represents a rising health problem in western countries. As currently no valid non-invasive method to differentiate between NAFL and NASH is available, and liver biopsies are still associated with elevated risk and cost the search for suitable serum biomarkers is pursued with high priority.

In the studies we have undertaken, miRNA expression with regard to their potential to predict the presence of NASH as serum biomarkers were in the focus of interest. The investigation of a panel of miRNAs and their appearance in serum brought new relevant findings for three of those candidates: MiR-122, -192 and -21.

Liver specific miR-122 has been associated with *de novo* lipogenesis and lipid trafficking[235, 236]. Also, a significant increase of miR-122 in the serum of NASH

patients has been identified with positive correlation to the stages of inflammation and fibrosis[235]. The inhibitory effect of miR-122 on the stellate cell activation and collagen deposition within the liver described by Li et al. is indicating a link between decreased miR-122 and fibrotic liver damage[237]. The analysis of human liver tissue showed a decreased expression of miR-122 in patients suffering from NAFLD[139]. On the serum level, Pirola et al. had shown an increased miR-122 appearance with the manifestation of NASH[97] as previously described for several other chronic liver diseases[279, 280]. The data this work is based on, obtained from tissue and serum could validate the described observations: An inverse correlation of miR-122, with a downregulation in liver tissue and its increased appearance in serum. The molecular basis of this observation is a described correlation between hepatocyte destruction and miR-122 export[142, 281, 282].

MiR-192 is a miRNA scarcely investigated in the context of NAFLD progression. It is known to be upregulated by TGFβ1[238], a key fibrogenic cytokine in hepatic stellate cells. In a study, already mentioned in respect to miR-122, Pirola et al. observed an increased miR-192 level in serum of NAFL and NASH patients, also suggesting a role of this miRNA in different pathologies within the metabolic syndrome[97].

The results of our studies could confirm the observations of Pirola et al.[97], indicating a significant correlation of miR-122 and miR-192 to the manifestation of NASH. Most importantly, significant differences for both miRNAs could be confirmed between NAFL and NASH patients and render these two as biomarker candidates. An interesting finding was the observation, that this difference was more prominent in

our cohort suffering from morbid compared to moderate obesity. In moderate obese patients, in spite of consistent direction of effects, statistical significance was not obtained. These observations might suggest a regulation of miR-122 and -192 dependent on the degree of obesity.

MiR-21 has been associated with several diseases before; a fact which has been already analysed critically in respect to its potential use as a prognostic marker[240]. It was also one of the first described oncomirs[245]. The new finding from this work now is a significant upregulation of miR-21 exclusively in patients suffering from NASH. The present data now indicate that miR-21 is further increased in inflammatory states of fatty liver disease since no impact of obesity status of patients was observed.

The positive correlation of miR-192 and -122 to CK-18 fragments and ALT levels indicate a release of these miRNAs by hepatocytes during pathophysiological states associated with cell membrane impairment. Roderburg et al. describes the increase of miR-122 in serum with apoptosis of hepatocytes caused by inflammatory damage of the liver[283] which might explain the positive correlation with CK-18 fragment levels, a described marker for hepatic apoptosis. Another new finding obtained from our study is the positive correlation between serum miR-21 expression and ALT level. These observations lead us to the hypothesis, that these miRNAs generally show an earlier increase than serum ALT in NASH patients; an observation which

has already found to be an advantage for miR-122 in the context of viral, drug- and alcohol-related liver disease[222, 284].

The aim of our study consisted in combining potential microRNA- and other biomarkers to increase the diagnostic performance in the discrimination of NASH from lower risk NAFL patients. Logistic regression analysis brought us to the conclusion to further analyse miR-122, -192, and -21 as potential biomarkers candidates. The former extensive investigations of CK-18 fragments as a biomarker candidate brought us to the decision to include its appearance as a reference together with serum ALT levels into our considerations[14, 92, 93]. In a simplified composite score model, the median was defined as critical threshold to discriminate a 'lower risk' from a 'higher risk' group for every single biomarker candidate. We found CK-18 fragment levels to have a significantly higher prediction potential compared to ALT shown in ROC analysis, which exceeds the minor predictive values in the existing literature[97]. The newly developed miRNA scoring system showed the same diagnostic performance in the discrimination of NASH as CK-18 fragment serum levels. Finally, the combination of the 3-miRNA expression panel with CK-18 fragments could even further improve the diagnostic performance.

In respect to the future impact of our observations, it has to be mentioned that 25% of miRNA/CK-18 detected NASH patients had normal ALT, which is also strengthening our hypothesis made earlier concerning an earlier diagnosis via miRNAs compared to ALT.

Serum miRNAs have been proposed as attractive diagnostic tools because of their minimal invasive nature and high stability in patients' serum[114]. The described results from our study are a promising step in the development of a reliable serum biomarker panel to identify patients "at risk" for NASH and thereby decrease the currently almost universal need of liver biopsies with potential complications[86].

5.4 Role of MiRNAs in the pathophysiological context of NAFLD

The development and progression of NAFL and NASH is not entirely understood on the pathophysiological level. The focus of the second study described in this work (see 4, Second manuscript) was laid on the expression of four miRNAs in either healthy livers and in different stages of fatty liver disease. This exploratory approach included also an analysis of regulative mRNA targets of these miRNAs and a first functional analysis in a cell culture model.

MiR-21 showed an upregulation following the onset of NAFLD in the liver biopsy cohort. This behaviour could also be observed by Vinciguerra et al., who described an upregulation in hepatocytes after unsaturated fatty acid treatment, mediated by NF-κBp65/ mammalian target of rapamycin (mTOR)-complex. The determined mechanism was a specific 3'- UTR binding of miR-21 to the PTEN messenger transcript, initiating its degradation[247]. Our observations in cultured hepatocytes stand on the first sight in contrast to these findings described in the literature. They

show a downregulation of miR-21 in the in vitro model. A possible explanation could be the longer incubation with oleic acid in our approach. As Vinciguerra et al. described a fast increase of miRNA expression after 24 hours with low concentrations of FFA, we suspect a fast alteration in the beginning followed by a decrease caused by the binding to PTEN transcripts[247] after 32 hours. Also possible would be an intracellular mechanism setting the miRNA free during cell degradation in the extracellular matrix as it is known for miR-122 [139, 235]. Not only the results obtained in this work show the enormous potential of miR-21 as a key mediator of MetS and NAFLD and suggest it to remain in the focus of scientific investigation[178, 249, 250].

The important role of miR-122 has been addressed earlier in this work. Cheung et al. described a highly significant downregulation of miR-122 in NASH patients[139]. This particular downregulation could be validated in the present study (4, Second manuscript). In addition to the validation of these results a significant difference between NAFL and NASH patients could be detected. Cheung et al. could describe a number of effects in the field of protein translation, cell proliferation, inflammation, apoptosis, oxidative stress and metabolism. The knowledge of an altered lipid metabolism caused by decreased miR-122[139] lead us to the hypothesis that functional regulation could be expressed by an upregulation of the miR target genes *JUN* (inflammation) and *FASN* (lipogenesis). As predicted an upregulation of miR-122 could be observed in human liver tissue of NAFLD patients but could not be replicated in the hepatocyte model. The significant difference in miRNA expression

between NAFL and NASH patients could not be detected at the level of potential mRNA target transcripts. An explanation would be the fact that often numerous miRNAs are targeting one single gene transcript at the same time with interfering effects[285]. Nevertheless, the correlation of the expression pattern between miR-122 and its mRNA targets in human liver tissue might be a hint for a regulative interaction.

MiR-223 was not only in the focus of interest in the context of hepatocellular and colorectal carcinoma in the past[253, 286] but also regarding cholesterol uptake and homeostasis[252, 287]. However, it has not been investigated in human fatty liver disease to date. As elaborately described in chapter 4, an upregulation of the miRNA could be observed in the human study cohort as also in the cultured hepatocyte model. These results are strengthened by the results of a study conducted by Shpyleva et al. describing an altered hepatic expression in a rodent model of fatty liver disease[251]. The fact that miR-223 is also targeting the investigated genes of interest *JUN* and *FASN* is another example for the fact the gene expression is not only regulated by one particular miRNA[285].

The rather newly described miR-638 has not been investigated in the context of NAFLD and MetS at all. It came into the focus of interest when it showed a different regulation in a pilot study comparing groups of NAFL and NASH patients (unpublished data). Of further importance, one of its predicted targets is perilipin5 (*PLIN5*), an essential mediator within the pathway of coating intracellular lipid

storage droplets as well as their protection from lipolytic degradation[267]. The prevention from lipolysis is a crucial step in the development of hepatocellular steatosis. The expression of perilipins is highly abundant to adipocyte and steroidogenic cells, as for instance hepatocytes[268-270]. In the extended context of liver diseases, there was no relevant data existing on miR-638, until very recently Kumar et al. showed its antiviral potential in the context of HBV infections[254]. Other studies showed different regulations in colorectal cancer patients[255, 256] but were lacking a final clarification of the underlying functional pathways. In this work the expected difference in expression within different stages NAFL vs. NASH could not be validated. However, a clear downregulation in the whole group of NAFLD patients compared to controls could be observed and a similar behavior appeared also in FFA treated cultured hepatocytes. In IHH cells a significant effect on *PLIN5* was observed for treatment with low concentrations of FFA, which might be evidence for a dependence on the degree of lipid storage. This clear effect on *PLIN5* was lacking in the tissue cohort, even though direction of regulation was as expected. The verification of these predicted target genes, suggesting a regulatory role in the context of metabolic manifestations or a potential contribution to NAFLD development represent one goal of further investigations within this field.

6 CONCLUDING REMARKS AND OUTLOOK

In the presented studies, we could underline the important role miRNAs are playing in the pathophysiology and the diagnostic context of NAFLD.

The obtained findings concerning the development of a reliable serum biomarker for the identification of NASH patients bear high potential for future improvement of clinical routine, particularly for early disease detection. The conclusion on the histological state of the liver presented by a non-invasive method fuel the assumption, that early detection and in turn treatment could be optimized by the use of miRNAs as additional biomarkers. The final establishment as a solid marker can only be obtained in multicentre studies with high numbers of enrolled patients.

The higher expression of miR-21 in NASH patients' serum leaves room for further investigation. An exclusive upregulation in this particular group is suggesting a potential role in the manifestation of the inflammatory state of the disease. As an upregulation of the miRNA could be also detected in liver tissue, a NASH specific release in the serum can also be suggested to be investigated with high priority. Also speculations may be allowed about a potential role in cell-cell communication. The high correlation of miR-122 and -192 in patient sera fuel the assumption about a potential link in regulation and leave space for further studies.

Together with preclinical functional studies the obtained results considering the four microRNAs differentially expressed in liver tissue of NAFLD patients and healthy controls give a clear hint on the high importance of miRNA regulation in the

pathophysiology of NAFLD. The newly described discrepancy of miR-122 expression in NAFL and NASH patients calls for further investigations in larger patient cohorts. An in-depth evaluation of miRNA and mRNA expression should bring new light in the regulative link between miR-122 and its targets genes.

Further planned activities within this field would be the complete analysis and evaluation of two already performed broad expression data assays investigating the miRNA and mRNA expression in the liver tissue of NAFLD patients on an unbiased basis. The obtained data of both assays is already subject of mathematical network and correlation modelling, creating a broad insight into regulation of NAFLD progression in liver tissue. With this *in silico* approach a description of the relation between potential regulators and their respective targets can be achieved. The calculated alterations in pathway processing represent the groundwork of further functional assays in an in vitro model upon availability.

The results of this work are giving clear evidence, that the investigated miRNAs are playing an important regulatory role in the context of NAFLD and give a hint on their influence on potential target genes. A final analysis also considering the full unbiased miRNA/mRNA expression assay data will bring light in the multi-regulated miRNA/transcript conjunction. Taking the outcome of these upcoming calculations together with the results presented within this work will build the essential basis for the final functional proof of the essential functional and pathophysiological role of microRNAs in the context of the development and progression of non-alcoholic fatty liver disease.

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09/2003 – 11/2010 **University of Heidelberg (Diploma Molecular Biology**)

- Thesis at the Institute of Human Genetics, University Hospital Heidelberg, group of PD Dr. Beate Niesler
    - Project 'Investigation of the link between genes of the serotonergic system and the aetiology of Irritable Bowel Syndrome'
- Additional Courses at the Faculty of Bioscience, Heidelberg (Molecular Biology, Cytology and Zoology)
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Work Experience

09/2010 – 01/2012 **University Hospital Heidelberg, Institute of Human Genetics, Division of Molecular Human Genetics, Group PD Dr. Beate Niesler**

- Genome Wide Association studies (GWAS); SNP - Genotyping in patient cohort studies.
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Awards

EASL Young Investigator bursary, EASL Monothematic Conference: Systems Biology of the Liver and Clinics face à face, Luxemburg (L) (February 2013)

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PUBLICATIONS

Article publication (Peer-reviewed manuscript)

Becker PP, Rau M, Schmitt J, Malsch C, Hammer C, Bantel H, Müllhaupt B, Geier A. (2015) Performance of serum microRNAs -122, -192 and -21 as biomarkers in patients with non-alcoholic steatohepatitis. PLoSONE 2015 Nov;10(11):e0142661.

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Abstract publication (Oral communication)

70th Annual DGVS Meeting: Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten, Leipzig (D):
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Abstract publication (Poster presentation)

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GASL Meeting: German Association for the Study of the Liver, Tübingen (D): The Pathogenesis of NASH: Focus on MiRNAs (January 2014)

AASLD: The Liver Meeting: 64th meeting of the American Association for the study of liver diseases, Washington DC (USA): MicroRNAs as Mediators in the Pathogenesis of Non-Alcoholic Fatty Liver Disease and Steatohepatitis (November 2013)

8th Integrative molecular Medicine-PhD-Retreat, Zurich Center for Integrative Human Physiology (ZIHP), Mariastein (CH): MicroRNAs in Metabolic Syndrome and Fatty Liver Disease (April 2013)

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